University of Alberta

Designing a point-of-care detection assay for tuberculosis

by

Susmita Sarkar

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Master of Science

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Fall 2011

Edmonton, Alberta

Abstract:

The current status of the disease of tuberculosis (TB) demands a rapid, simple and inexpensive point-of-care detection. This study reported the designing of an enzyme linked immunoassay that could detect the antigen lipoarabinomannan (LAM), found to be present in different body fluids of the infected individuals.

Initially, a bispecific monoclonal antibody was developed. It was subsequently used to design the sandwich immunoassay using the swabs. The limit of detection for spiked synthetic LAM was found to be 5.0 ng/ml (bovine urine), 0.5 ng/ml (rabbit serum) and 0.005 ng/ml (saline) and that for bacterial LAM from *M. tuberculosis* H37Rv was found to be 0.5 ng/ml (rabbit serum).

Finally the assay was evaluated using the stored clinical samples collected from the TB patients. In addition to good sensitivity, the end point could be read visually within two hours of sample collection.

Preface

The work described in this thesis was presented and accepted in several conferences. A concise version of Chapter 2-4 had been submitted in form of a research article in a scientific journal. Another short communication, describing the work mentioned in the Chapter 5, is under preparation.

Conference presentation:

- **Susmita Sarkar**, Lily Tang, Dipankar Das, Todd L.Lowary and Mavanur R Suresh. Development of a bispecific monoclonal antibody based assay for detecting TB antigen. At the annual general meeting of Alberta Ingenuity centre for Carbohydrate Science – Banff, Alberta, 2009.

- **Susmita Sarkar**, Lily Tang, Dipankar Das, Todd L.Lowary and Mavanur R Suresh. Detection of a tuberculin antigen using monoclonal antibody based immunoassay. At the Faculty Research day, University of Alberta – Nov-2009.

- **Susmita Sarkar**, Lily Tang, Dipankar Das, Todd L.Lowary and Mavanur R Suresh. Development of immunoswab assay for detecting tuberculosis. At the annual general meeting of Alberta Ingenuity centre for Carbohydrate Science – Banff, Alberta, 2010.

- **Susmita Sarkar**, Lily Tang, Dipankar Das, Todd L.Lowary and Mavanur R Suresh. Development of point of care assay for tuberculosis. At the annual meeting of ESPID, Nice, France. 2010.

- **Susmita Sarkar**, Lily Tang, Dipankar Das, Todd L.Lowary and Mavanur R Suresh. Designing a point of care assay for tuberculosis by using bi-specific monoclonal antibody. At the Faculty Research day, University of Alberta – Nov-2010.

List of Publications:

- Review Article: **Susmita Sarkar,** Mavanur. R. Suresh. An overview of tuberculosis chemotherapy – a literature review. *J Pharm Pharm Sci,* 2011, 14 (2): 148-161.

- Research Article: **Susmita Sarkar,** Xinling. L.Tang, Dipankar Das, Todd. L. Lowary and Mavanur. R. Suresh. A rapid and inexpensive swab-based point-of-care detection assay for tuberculosis (Submitted to journal).

- Book Chapter: Archana Parashar, **Susmita Sarkar**, Advaita Ganguly, Saikiran Sharma and Mavanur R. Suresh, 2011 Bispecific Antibodies for Diagnostic Applications. Ed. Dr. Roland Kontermann (U of Stuttgart), Springer, Heidelberg. (Editing in progress).

- Research Article: **Susmita Sarkar,** Dipankar Das, Todd. L. Lowary and Mavanur. R. Suresh. A synthetic carbohydrate antigen shows promising potential for a tuberculosis vaccine *via* dendritic cell targeted pathway. (Manuscript under preparation).

Acknowledgement:

At the beginning, I want to thank my supervisor Dr. M. R. Suresh for all his support, encouragement and able mentorship. His constant encouragement made it possible for me to finish this journey. I also want to convey my thanks to my committee members Dr. R. Loebenberg and Dr. T. L. Lowary for their able guidance, suggestions and time. Dr. Lowary and his group also helped us to procure various materials used in this study.

Thanks are also due to entire Suresh team who not only helped me to learn various techniques used in this study but also provided a wonderful and friendly atmosphere in the lab. I really enjoyed my time because of them.

I also want to thank my funding agency the Alberta Ingenuity Centre for Carbohydrate Science (AICCS), Alberta, Canada. The credit is also due to all the staffs of the faculty of Pharmacy and Pharmaceutical Sciences.

Finally I want to thank my family (my parents, sister and brother) for all their support and trust on me. Thank God for giving me chance to work, share and live with these wonderful people.

Table of Contents

Chapter I. Introduction

Chapter II. Expansion, purification, characterisation & biotin labelling of CS-35 MAb

Chapter III: Production and characterisation of the bsMAb

Chapter IV: Designing the immunoassay

Chapter V: Evaluation of the assay with the clinical samples

List of Tables:

List of Figures:

List of Symbols and Abbreviations:

µg	microgram
µg/ml	microgram/millilitre
Ab	Antibody
Ag	Antigen
AM	Alveolar macrophages
AP	Alkaline phosphatase
β –ME	β-mercaptoethanol
BSA	Bovine serum albumin
BSA-Hex	Bovine serum albumin conjugated hexaarabinofuranoside
bsMAb	Bispecific monoclonal antibody
CAb	Capture antibody
cell/ml	cell per millilitre
CO_2	Carbon dioxide
DAb	Detection antibody
DBSA	Dialyzed bovine serum albumin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay

EME	Established market economics
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAM-HRPO	Goat anti-mouse IgG conjugated to horseradish peroxidase
h	hour
HAT	Hypoxanthine-aminopterine-thymidine
Hex	Hexaarabinofuranoside
HGPRT	Hypoxanthine-guanine-phosphoribosyl transferase
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IC	Immune complex
IgG	Immunoglobulin class G
LAM	Lipoarabinomannan
Lep-LAM	Lipoarabinomannan from *Mycobacterium leprae*
MAb	Monoclonal antibody
m-APBA	*m*-Amino phenyl boronic acid agarose
MDR	Multi drug resistant
mg	milligram
min	minute

ml	millilitre
Mtb	*Mycobacterium tuberculosis*
NAAT	Nucleic acid amplification test
ng	nanogram
ng/ml	nanogram/millilitre
NTM	Non Tuberculin Mycobacteria
pAb	Polyclonal antibody
PBS	Phosphate saline buffer
PBST	Phosphate saline buffer containing 0.05% tween-20
PEG	Polyethylene glycol
POC	Point of care
PSG	Penicillin-streptomycin-glutamine
ROW	Rest of the world
RPM	Revolution per minute
RS	Rabbit serum
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SFM	Serum free media
SM	Skim milk
Smeg-LAM	Lipoarabinomannan from *Mycobacterium smegmatis*
S/N	Signal / Noise ratio

SSM	Sputum smear microscopy
St-AP	Streptavidin tagged alkaline phosphatase
St-HRPO	Streptavidin tagged horseradish peroxidase
TB	Tuberculosis
TB-LAM	Lipoarabinomannan from *Mycobacterium tuberculosis*
TMB	3,3',5,5'- Tetramethylbenzidine
TRITC	Tetramethylrhodamine isothiocyanate
TT-Hex	Tetanus toxoid conjugated hexaarabinofuranoside
WHO	World Health Organisation
XDR	Extremely drug resistant

Chapter I: Introduction

1.1. The past, present and future of Tuberculosis

Tuberculosis (TB), one of the major global public health threats, is claiming almost 4600 lives per day worldwide as 1.68 million deaths were estimated due to TB on 2009 (WHO annual TB report 2010). The scientists from different parts of the globe have tried to figure out the origin of this deadly disease by exploiting the DNA isolation and amplification techniques. They were even able to detect the presence of TB from the ancient human remains. Initially it was thought that humans adopted TB from the infected animals as a result of domestication which occurred about 10,000 years ago (Cockburn, 1964). But later it was found that humans had TB even before domestication (Rothschild *et al*., 2001). Some researchers have even extended the origin of TB almost 3 million years ago (Gutierrez *et al.*, 2005).

The current spread of TB can be estimated by the fact that almost one third of the world population are carrying it in the latent form. According to the World Health Organisation (WHO) report, almost 9.4 million incidents of TB were reported in 2009 with approximately 1.68 million deaths (WHO global TB report 2010). The global spread of TB is shown in Figure 1.1. Almost 80% of the TB cases were reported from the south-east Asia and sub-Saharan Africa so they come under the category of high burden country. The emergence of multi drug resistant (MDR) and extremely drug resistant (XDR) strains of the bacteria along with the human immune deficiency virus (HIV) had further worsened the disease management. TB is the leading killer for the HIV infected people. The global distribution of HIV for TB infected patients was shown in Fig 1.2.

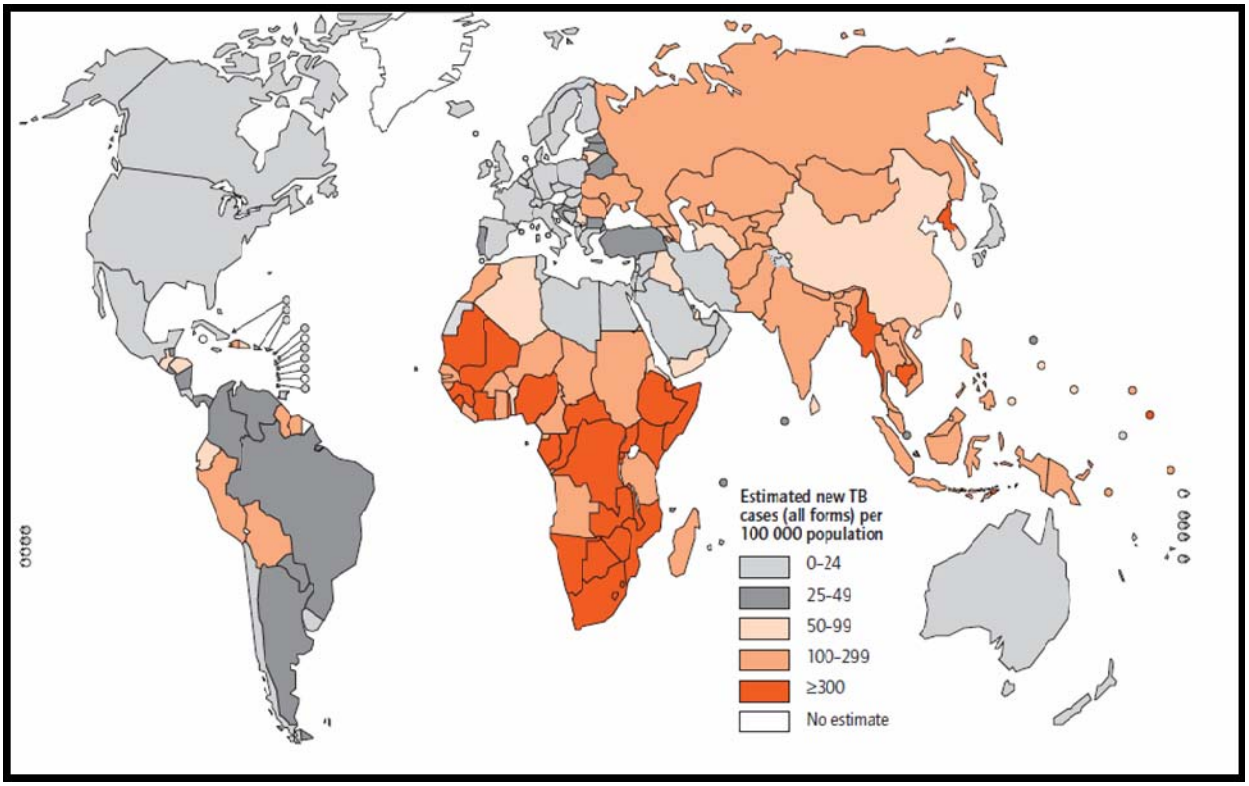


Fig 1.1. The estimated global TB incidences rates. The figure source: WHO annual TB report 2009.

In order to control the global burden of the disease, WHO had recommended certain plans which are collectively known as the Stop TB strategy. The target is to reduce the global prevalence and mortality of TB to 50% of that of 1990 value by the end of 2015 and in the long run reduce the TB incidence to 1 case per million per year by 2050 (WHO annual TB report 2010). However, the rapid emergence of drug resistant bacteria is making the control much more difficult and complex. In 2009, estimated 250,000 cases of MDR-TB were notified. Till July-2010 at least one confirmed XDR-TB case was reported from 58 countries and territories (WHO global TB report 2010).

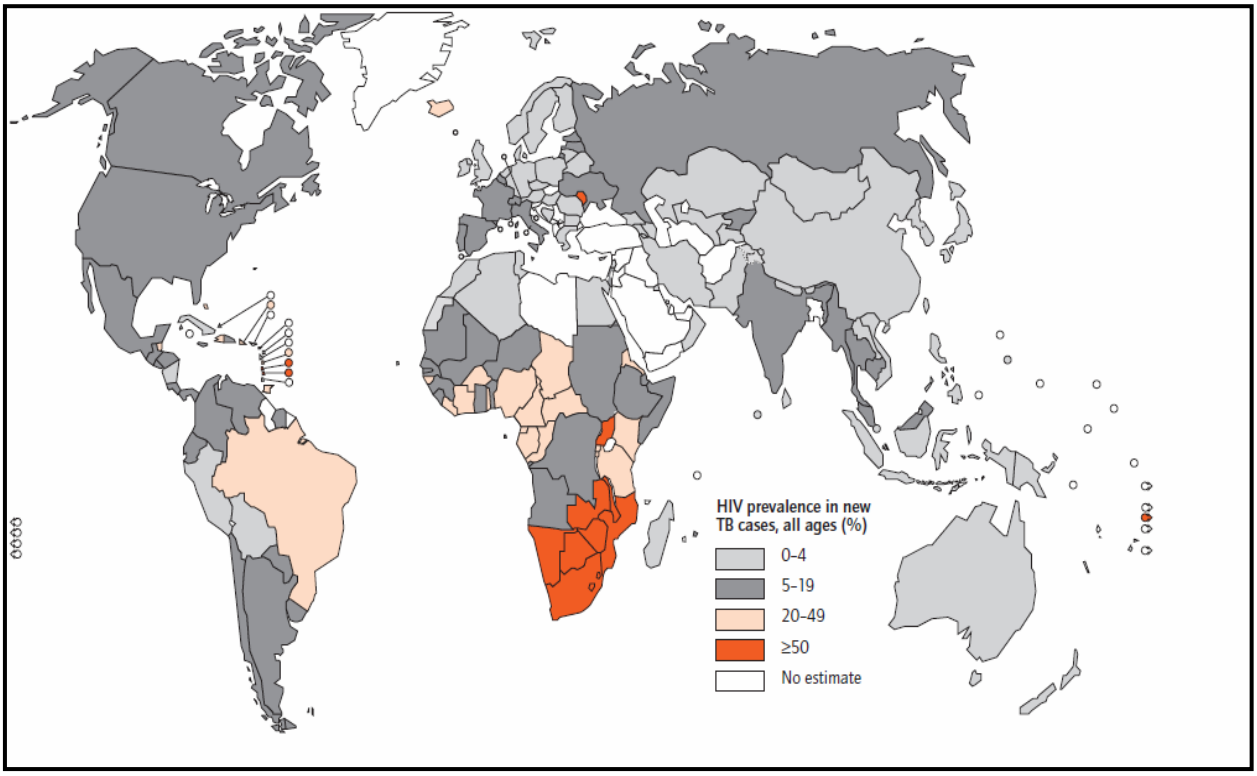


Fig 1.2. The estimated HIV prevalence in the new TB incidences rates. The Figure source: WHO annual TB report 2009.

1.2. The pathogenesis of tuberculosis

Mycobacterium family has over 60 species but only few of them can cause diseases in human like *Mycobacterium tuberculosis* (Mtb), *Mycobacterium leprae, Mycobacterium africanum* and *Mycobacterium avium*. Mtb can exist in the human body for a long time without showing any clinical symptoms. This stage is called the latent stage of the disease. Once the host's immune system becomes weakened, either because of age or concurrent disease, then the bacteria attains virulent or active form. TB is highly contagious during the active stage and the primary route of infection is by the pulmonary system. The live bacteria were found to be present in the sputum and cough droplets of infected patients. The disease can be contracted by inhaling as low as 10 bacteria (Lipman *et al.* 2005). After entering through the nasal route, the bacteria are mainly captured by the alveolar macrophages (AM) (Leemans *et al*., 2001). In healthy individuals, the bacteria remain confined within the AM under the strong immune surveillance and do not show any disease symptoms. But the bacteria can survive within this AM for as long as 10-12 years and the exact mechanism of bacterial survival in this constrained condition is still not well known. There are reports that bacteria do secrete some antigens that are capable of manipulating the host immune system (Ellner 1997, Briken *et al.* 2004; Jozefowski *et al.*2008).

The bacteria from this latent stage can rapidly regain virulence once the immune system of the host become weak and the bacteria invade the pulmonary system. Though TB mainly affects the pulmonary system but in the severe immune compromised patients it can also affect the lymphatic system, gastrointestinal system, skeletal system, genitourinary system, central nervous system etc (Golden *et al.* 2005). The extrapulmonary form of TB is spreading in both

developed and developing countries and it is quite common for HIV and TB co-infected population and, in fact, more than 50% of the HIV-TB population have the extrapulmonary TB (Pitchenik *et al.* 1984, Sharma *et al.* 2004). The spread of TB in different body parts is summarised in Fig 1.3.

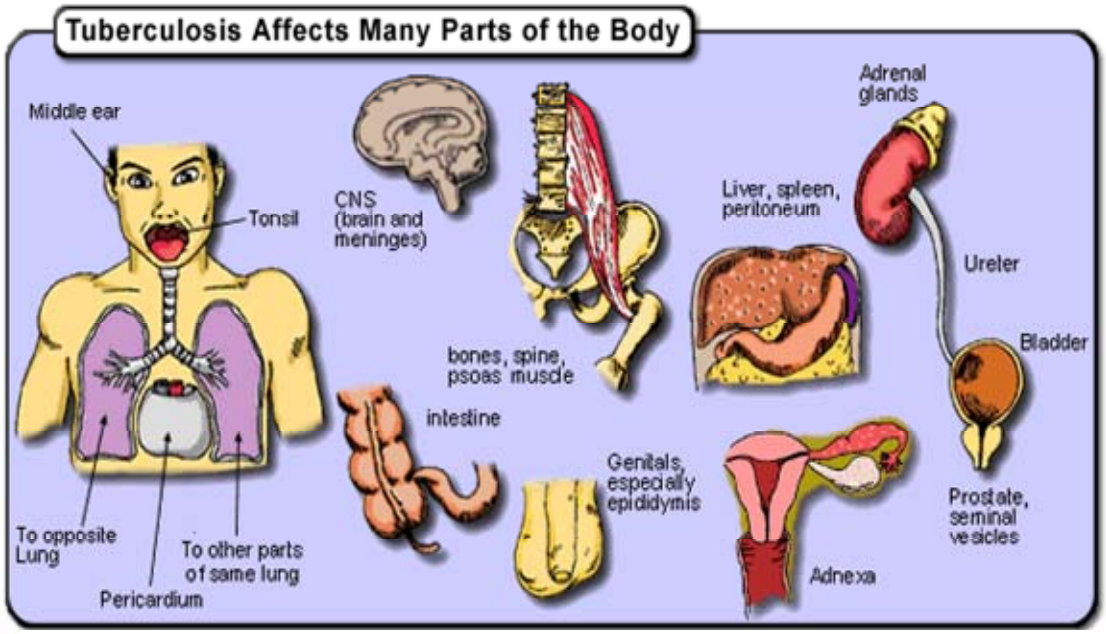


Fig 1.3. Different parts of the body that are affected by TB. Picture Source: http://www.indiastudychannel.com/attachments/Resources/57983-13145-rccm_tuberculosis_body.gif

The TB bacteria exploit the weak immune system of the host to gain virulence and hence they belong to the group of opportunistic pathogens. Because of this, the paediatric, elderly, HIV positive population and the population with other concurrent conditions like diabetes, drug abuse, malnutrition etc, are regarded as the high risk groups for TB (Sharma *et al.* 2005). In fact, TB is the leading cause of death for HIV affected people (WHO annual TB report 2010 and Granich *et al*. 2010).

The early stage of TB is characterised by mild coughing, weakness, fever, night sweat and weight loss. The advanced stage of the disease symptom includes coughing up of sputum with or without blood, shortness of breath and chest pain. (Ferguson and Schwarz 2010)

1.3. TB bacteria

The main causative agent for TB is the *Mycobacterium tuberculosis* (Mtb), which is a rod shaped bacteria having 2-4 μm length and 0.2-0.5 μm width (Online text book of bacteriology). The bacteria were first discovered by Robert Koch in 1882 on the day of 24th March and for this he was awarded the Nobel Prize in 1905. To commemorate his great discovery, every year the World TB day is celebrated on 24th of March.

Mtb is an aerobic bacteria but its survival mechanism inside the human granuloma is still not well understood. In the human host, the TB bacteria can simultaneously remain in three distinctly different replication stages. The first one replicates rapidly and resides in well oxygenated cavities. The second one has moderate replication capability and resides in poorly oxygenated caseous lesions and the third population is almost dormant with occasional replication in acidic environments (Wecker *et al*. 2010). This third group of bacteria is responsible for latent and relapse of TB. Because of this unique capability of the bacteria, the treatment of TB consists of multiple antibiotics with longer duration of treatment. Different antibiotics target different population of the TB bacteria (Mitchison, 1979).

1.4. Current diagnosis

TB can be treated with multi drug treatment but the timely initiation of the treatment is vital to get the desired result. For that a specific and sensitive detection of the disease is important. There are different tests available to diagnose TB, but all have their own merits and demerits. Some of the commercially practised detection test and techniques are discussed in the following sections.

1.4.1. Chest X-ray

Since TB primarily affects the pulmonary system, a chest X-ray is an easy option to detect TB. The technique gives a quick result and easy to perform. But it requires instrument, technical knowledge and also electricity to operate it. These things are difficult to imagine in a remote health care center in Sub Saharan Africa, which shares the key load of the global TB burden. Moreover, many other pulmonary conditions like pneumonia, fungal infections etc. can give identical chest radiograms. So detection on the basis of chest X-ray is basically non specific but still it is practiced in some parts of the world because the result can be obtained very fast and the process is easy to perform (TDR publication, 2006).

1.4.2. Sputum Smear Microscopy (SSM)

The SSM is the widely used detection method for TB. After collecting the sample, the sputum smear is prepared on glass slides then it is fixed, stained and dried. Finally, the result is read under microscope. The result can be obtained within 2-3 hrs and it is also cheap to perform. SSM performs well in the highly infectious cases and in high burden countries but its sensitivity

is poor (Menon *et al*. 2000). It can detect the cases when the bacterial load is at 10^4 bacilli/ml of the sample so the smear positive patients are extremely infectious.

The performance of SSM is poor with the HIV infected patients. A recent review mentioned that average sensitivity of the SSM is <60% for immunocompetent patients and the figure is much lower for immunocompromised patients (Perkins and Cunningham 2007). This is perhaps because of the fact that during HIV and TB co-infection, the lower lobe of the lung was found to be more involved and hence the bacterial concentration is reduced in the sputum (Colebunders and Bastian 2000). Another problem with the SSM is that it is sometimes difficult to collect the required sputum sample from the elderly and paediatric population, the two important groups of TB victim (Steingart *et al*. 2006). The test also requires dedicated facilities.

1.4.3. Culture method

The culture method for detecting the TB is still regarded as the gold standard for detection. It can detect the disease when the bacterial load is as low as 10 bacilli/ml of sputum. The drug susceptibility test and identification of the bacterial strain can also be performed simultaneously by this method. The major drawback of this method is the time required to get the result. Two to six weeks are needed to report the result, the time may be reduced to 1-4 weeks by using improved media but that adds up the cost for the assay. The technique also requires dedicated culture facility.

1.4.4. Nucleic Acid Amplification Test (NAAT)

The NAAT is comparatively new technique to detect TB. The test involves amplification of bacterial DNA or ribosomal RNA via polymerase chain reaction (PCR). The test is very specific and sensitivity was found to be 90% in sputum smear positive cases and 60-70% in smear negative cases (Clarridge *et al*., 1993; Abe *et al*., 1993). However, the huge cost and the technical sophistication of the assay limit its use in the resource constrained settings of the high burden countries.

1.4.5. Tuberculin skin test

The tuberculin test is an immunological test consists of injecting the purified protein derivative (PPD) obtained from the heat killed bacteria (TDR publication, 2006). If a person is infected with the disease then the resulting immune response can be detected in the form of an induration. The result is interpreted by measuring the induration and also considering the patients other medical conditions. The latent TB can also be detected by this method. But this test gives false positive result in the BCG vaccinated population and as well as in the population carrying the non pathogenic mycobacteria. The test also performs poorly in the severely immunocompromised patients.

1.4.6. Interferon gamma assay

This is another immunological test that measures the T-cell response. The cytokine response is measured against two important protein antigens that are highly specific for *Mycobacterium tuberculosis*. The latent TB can also be detected by this assay. Though the assay is very specific but the reliability of the result was found questionable (Madariaga *et al*., 2007) and the technique is expensive and requires technical sophistication.

On the basis of the above discussion, it is clear that none of the available detection technique can be used as a point of care (POC) assay for diagnosis of TB.

1.5. Monospecific and Bispecific monoclonal antibody

1.5.1. Background & Discovery

Antibodies, also known as immunoglobulins, are a class of protein molecules that are secreted by the plasma cells to neutralize and eliminate invading pathogens. The antibodies are integral part of the adaptive immune system and they are generated by the B cells or plasma cells in response against the pathogen (Kindt et al. 2007). The part of the antibody that binds to an antigen is called the paratope of the antibody and the natural antibodies have two identical paratopes and that is why they are also monospecific. The part of the antigen that is recognised by the antibody is termed as epitope of the antigen. Most of the pathogenic antigens have

multiple epitopes on its surface that may or may not be identical. As a result, the natural antibody response is always heterogeneous as it is directed towards the various epitopes on the antigen. This kind of heterogeneous response is called polyclonal antibody (pAb). This type of pAb response can cover a wide range of protection against a pathogen. However, they show cross reactivity in the diagnostic application (Berry *et al.* 2005). Another disadvantage of this natural antibody response is that they have a certain biological life span. The average half life of peripheral B cells is 5-6 weeks while the other varieties have a life span of few days (Fulcher *et al.* 1997). The life span decreases further in *in vitro*.

These two disadvantages, cross reactivity and short life span, were solved by the Nobel Award winning discovery of the hybridoma technology by G. Kohler and C. Milstein (Kohler and Milstein, 1975). The hybridomas are formed by fusing specific antibody producing B cells with myeloma cells. The resultant hybrid cell line inherits the antibody secretion capability from the parent B cell and at the same time it inherits the capability of continuous growth from the myeloma cell. This immortal hybrid cell lines can easily be cultured *in vitro* in suitable nutrient media. Since the hybridoma cell lines are generated by fusing one B cell with one myeloma cell so the secreted antibody will always have a single specificity, identical to the parent B cell. Due to this single specificity they are also called monoclonal antibody (MAb). The discovery of MAb generation greatly revolutionised the application of antibody in therapeutic as well as in diagnostic area.

The combination of a B cell with a myeloma cell produces an antibody having specificity towards the same epitope of an antigen, in fact, the hybridomas secrete antibodies that are bivalent (two paratopes) and monospecific (binds to the same epitope). A second generation

of hybridoma technology, called the quadroma technology, was subsequently developed and since then it has been widely used in various *in vitro* and *in vivo* applications (Milstein *et al*. 1983, Suresh *et al*. 1986a, Suresh *et al.,* 1986b). The antibody secreted by the quadroma cell line can simultaneously bind to two different epitopes of a same antigen or of two different antigens. So, they are called bispecific monoclonal antibody (bsMAb) (Das and Suresh 2005).

1.5.2. Production of Monoclonal antibody

The antibodies are generated by the B cells. Once the innate immune system fails to control the infection by an invading pathogen then the adaptive immune system starts to fight the infection. The humoral part of the adaptive immune system is mainly taken care of by the B cells. The antibodies or the immunoglobulins are the surface molecule on the B cells and a single B cell can express multiple numbers of antibodies but all having the same specificity. The polyclonal response is generated as a combined effect of various B cells. In response to a pathogen or any foreign antigens the B cells differentiate into plasma cells which secrete the antibody (Kindt et al. 2007). But as discussed before, these B cells have certain life span and also the polyclonal nature of the response often times create cross reactivity when used in diagnostic purpose. The monoclonal antibody (MAb) can solve both the problems.

To generate the hybridoma for research purposes, the mouse is largely used to generate the immune response against the antigen of interest. The antigens are first injected in mice either in suitable buffer solution or with an adjuvant for better immune response. The antibody response is measured by doing the enzyme linked immunosorbent assay (ELISA) with the blood taken from the tail vein of the immunized mice. A booster dose is administered if required and the mice are euthanized and subsequently, spleens are collected aseptically. The

spleens are mashed and a single cell suspension is made. The B cells obtained from the spleens were mixed with the myeloma cells which are mouse SP2/0 cell lines. This specially engineered cell line lacks the antibody secretion capability and it also lacks the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT). The fusion is performed by using polyethylene glycol (PEG). The fusion process is quite random and can give rise to a mixture of non-fused, homo fused (either two B cells or two myeloma cells) and hetero fused (one B cell with one myeloma) cell lines of which the last one is of importance since they are the hybridomas formed by fusing one B cell with one myeloma cells. To select the hybridoma cell line from the rest, a selection medium is used. The hypoxanthine-aminopterine-thymidine (HAT) medium is used to culture the cells. The SP2/0 cells, either non-fused or the homo fused, cannot survive in this media as they do not have the required enzyme. The B cells could survive in this media but the non-fused and homo fused B cells cannot survive long under *in vitro* conditions. So they will eventually die. Only the hybridoma cell line can survive in this media as it inherited the required enzyme to survive in HAT media from the parent B cell and at the same time it acquired the gene for infinite growth or immortality from the other parent, SP2/0. After culturing the cells for 10-12 days their activity is checked using the ELISA. The positive clones are selected and cloned by the limiting dilution method for 2-3 times for getting the stable clone. The generation of a MAb is schematically shown in Fig 1.4.

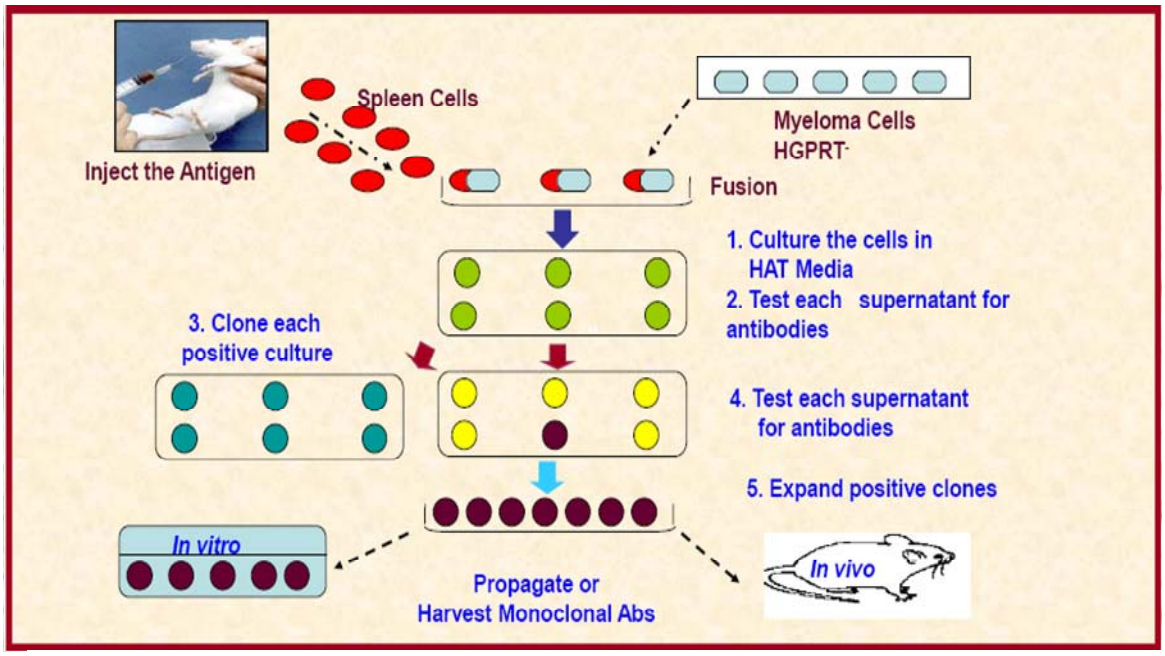


Fig 1.4. The schematic representation of the MAb preparation.

1.5.3. Production of bispecific monoclonal antibodies

The bispecific monoclonal antibodies (bsMAb) are the second generation MAb that has specificity towards two different antigens. The bsMAbs are obtained by fusing two different hybridomas and that is why they are called a quadroma. The bsMAbs are made up of two different heavy and light chains, each of the heavy and light chain are contributed by each of the parent hybridoma. The bsMAb construct is schematically shown in Fig.1.5. Nisonoff and Rivers first generated the bispecific antibody by a chemical method in 1961 (Nisonoff and Rivers, 1961) but it was of polyclonal variety since during that time hybridoma technology was not discovered. After the discovery of the hybridoma technology in 1975 (Kohler and Milestein, 1975), the idea of bsMAb was introduced in 1983 (Milestein and Cuello, 1983).

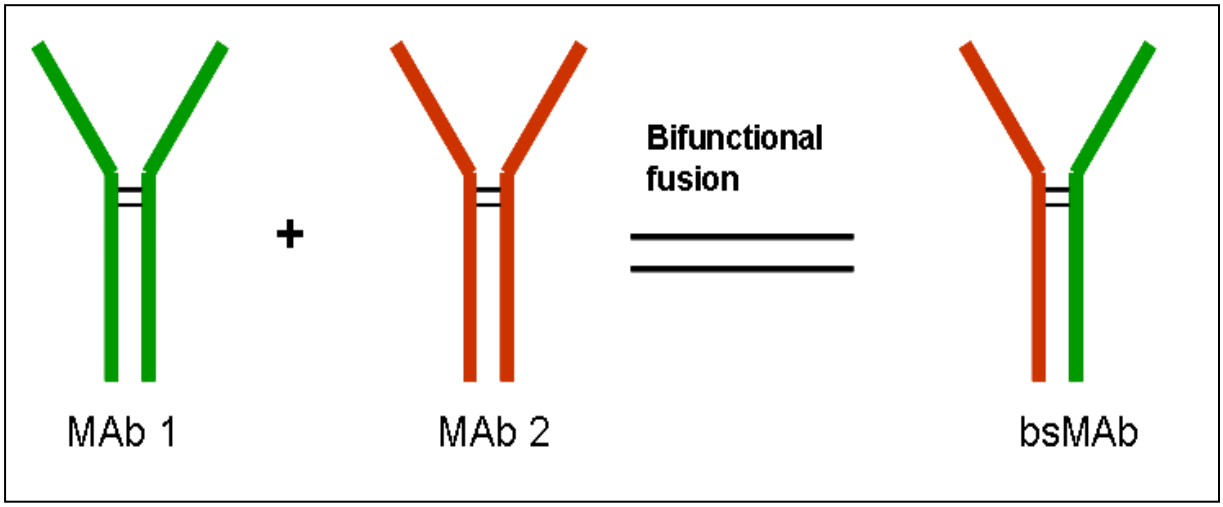


Fig 1.5. The schematic representation of the bsMAb construct.

There are mainly three different methods for generating the bsMAbs – a) chemical coupling, b) somatic hybridization and c) recombinant DNA technology.

In the chemical coupling methods, the association of two different antibodies are achieved by two different ways. One way of doing this is by reducing the inter-heavy chain disulphide bond present in the two parent antibodies and then one half from each parent is re-associated via a cross linker. Reagents like 5,5`-dithiobis (2-nitrobenzoic acid) (DTNB) and *o*-phenylenedimaleimide (O-PDM) have been employed to make bsMAbs (Brenner *et al*., 1985; Glennie *et al.,* 1987; Shalaby *et al*., 1992 and Doppalapudi *et al.,* 2010). Another way of making the bsMAb is to introduce a reactive group on one antibody and then allow it to react with the other. This type of coupling reaction causes random association. One such chemical is N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), which had been used to prepare bsMAbs (Segal *et al*., 1988; Van Dijk *et al.*, 1989 and Jung *et al*., 1991). The chemical coupling methods

are easy and fast to perform. The yields are usually high but due to chemical manipulation the antibodies may get denatured or inactivated (Cao and Suresh 1998).

The somatic hybridization can also be achieved by two different ways – either by drug selection method or by dual fluorescence selection method. In the drug selection method, one of the hybridomas is made to be resistant towards an antibiotic and the other is made to be resistant towards a second antibiotic. The fusion between these two drug resistant hybridomas is performed as per the hybridoma fusion (Kohler and Milstein, 1975) and the fused cells are cultured in the suitable nutrient media containing both the antibiotics. The presence of both the antibiotics will ensure the survival of the heterofused cell only as none of the parent hybridomas are resistant to both the antibiotics so they will die along with the homofused ones. De Lau *et al.* have used this kind of fusion to generate the bsMAb (De Lau *et al.,* 1989). This type of somatic fusion is usually quite labour and time intensive. Another type of somatic fusion can also be achieved by using two different fluorophores like fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC). Here one hybridoma is labelled with FITC and the other with TRITC. Then the labelled cells are fused as before and the fluorescence activated cell sorting (FACS) is performed to select the dual labelled cells. This type of fusion is described in detail in the subsequent chapter. Many researchers used this technique to obtain the bsMAb (Krrutz *et al*., 1998; Tang *et al*., 2004; Das and Suresh, 2005 and Kammila *et al*., 2008). This method is very fast; however, the major challenge in this process is the purification of the bispecific protein and it is generally done by double affinity column purification.

The third type of hybridization is performed by the genetic manipulation. The most widely used technique is to express two different single chain antibodies *via* a linker molecule

and several studies have used this technique to generate the bsMAb of their choice (Winter and Milstein, 1991; Hollinger *et al*., 1993; Kranz *et al*., 1995; Schmidt *et al*., 1996; Thirion *et al*., 1996; Das and Suresh, 2005; Wang *et al.,* 2009).

1.5.4. Applications of Monoclonal and bispecific antibodies

1.5.4.1. Applications of MAb:

The discovery of MAb has revolutionised the field of biomedical research. Due to its excellent specificity towards a particular epitope of an antigen it has been widely used in diagnostic as well as in therapeutics. The use of MAb in the diagnosis reduces the chance of cross reactivity because of its high specificity. Since the MAbs target particular one surface epitope on the pathogen so they can distinguish between structurally related pathogens. The MAbs can easily be conjugated chemically with fluorophore, enzyme and biotin. Plenty of reports are available in literature regarding the use of MAb in diagnosis of infectious as well as non infectious disease like cancer (Kreutz and Suresh 1997; Gómez *et al*., 1997; Irmen and Kelleher 2000; Niikura *et al*., 2001; Syrigos *et al*., 2004 and Chiang *et al.,* 2010). The commercially available rapid pregnancy detection kit also includes MAb that can recognise the human chorionic gonadotropin hormone (pregnancy marker found in urine) (Bioquant hCG ELISA kit).

Apart from diagnosis, the MAbs are widely used for therapeutic purposes. The excellent specificity of the MAb makes it a useful tool to target specific over expressed markers on the cell

surface during the disease condition. They are also important to manipulate the immune system during tissue or organ transplant procedure. A list of MAbs that are approved by the Food and Drug Administration (FDA) is shown in Table 1. The MAbs raised against different cell surface markers are also employed in drug delivery system to ensue specific targeting of the drug at the required organ or cell. This type of strategy is particularly important for the delivery of cancer drugs (Akasaka *et al.,* 1988; Calabrò *et al*., 2010 and Ascierto *et al.*, 2010) since this type of delivery ensures specific drug delivery thereby reducing the dose and the related systemic toxicity.

Fluorophore and radioisotope - tagged MAbs are being widely used to map the expression of different cell surface markers under *in vitro* as well as *in vivo* conditions in the presence or absence of some chemicals of interest. Sometimes this can be achieved through non-invasive technique i.e. without sacrificing the test animal. That aids our research to find a potential drug or vaccine candidate.

Table 1.1: FDA approved therapeutic monoclonal antibodies (They are arranged according to their year of approval, starting from 2008. The informations were collected from Waldmann T A, 2003 and Wikipedia website)

Antibody	Brand name	Type	Indication	Approved
Certolizumab pegol	Cimzia	Humanized	Crohn's disease	**2008**
Eculizumab	Soliris	Humanized	Paroxysmal nocturnal hemoglobinuria	**2007**
Natalizumab	Tysabri	Humanized	Multiple sclerosis and Crohn's disease	**2006**
Panitumumab	Vectibix	Human	Colorectal cancer	**2006**
Ranibizumab	Lucentis	Humanized	Macular degeneration	**2006**
Bevacizumab	Avastin	Humanized	Colorectal cancer, Age related macular degeneration	**2004**
Cetuximab	Erbitux	Chimeric	Colorectal cancer, Head and neck cancer	**2004**
Omalizumab	Xolair	Humanized	Mainly allergy-related asthma	**2004**
Tositumomab	Bexxar	Murine	Non-Hodgkin lymphoma	**2003**
Adalimumab	Humira	Human	Several auto-immune disorders	**2002**
Efalizumab	Raptiva	Humanized	Psoriasis	**2002**

Ibritumomab tiuxetan	Zevalin	Murine	Non-Hodgkin lymphoma	**2002**
Alemtuzumab	Campath	Humanized	Chronic lymphocytic leukemia	**2001**
Gemtuzumab ozogamicin	Mylotarg	Humanized	Acute myelogenous leukemia	**2000**
Basiliximab	Simulect	Chimeric	Transplant rejection	**1998**
Infliximab	Remicade	Chimeric	Several autoimmune disorders	**1998**
Palivizumab	Synagis	Humanized	Respiratory Syncytial Virus	**1998**
Trastuzumab	Herceptin	Humanized	Breast cancer	**1998**
Daclizumab	Zenapax	Humanized	Transplant rejection	**1997**
Rituximab	Rituxan, Mabthera	Chimeric	Non-Hodgkin lymphoma	**1997**
Abciximab	ReoPro	Chimeric	Cardiovascular disease	**1994**
Muromomab-CD3	Orthoclone OKT3	Murine	Transplant allograft rejection	**1986**

1.5.4.2. Applications of bsMAb:

The bsMAbs have found applications in almost all the fields where MAbs are used. The bsMAbs have extra advantage of binding to two different antigens. The two different paratopes of the bsMAb can be selected based on its application. For example, in diagnostic applications one paratope is generally for an enzyme like alkaline phosphatase (AP) or horseradish peroxidase (HRPO) or *β*-galactosidase and other is for the respective disease marker. For therapeutic applications, one paratope is selected to carry the therapeutic agent, while the other can bind to the specific marker at the required delivery site (either organ specific or cell specific marker) (Mabry and Snavely 2010).

The enzyme tagging of bsMAb enables to detect the presence of target antigen in the sample based on colour development, subsequent to the addition of corresponding substrate. The immunoassay, employing the bsMAb, has some extra advantages over that of the assay using conjugated monoclonal antibody. Firstly, the chemical conjugation of the MAb is a random process where the combination ratio between the MAb and the conjugating chemical (biotin or any enzyme like HRPO) is not fixed. But the bsMAb has two different paratopes, one of which is dedicated towards the antigen of the targeted disease and the other paratope binds to a signalling molecule like an enzyme, HRPO. So the combination ratio between the bsMAb and the enzyme is always 1:1, which helps to get a clear background. Secondly, due to the random nature of this chemical conjugation, the site of conjugation is also not fixed and as a result it may block the antigen binding site on the MAb, which leads to reduced sensitivity. Finally, during the purification of bsMAb, it is already tagged with the signalling enzyme during the purification step (Bhatnagar *et al.,* 2008). Hence an extra step of enzyme addition and the subsequent

washing steps can be avoided in the assay involving bsMAb. Because of these added advantages, bsMAbs found wide application in diagnosis (Cao *et al*., 1998; Lie *et al.,* 2003; Tang *et al.,* 2004; Guttikonda *et al*., 2007; Kammila *et al*., 2008).

Due to presence of two different paratopes in bsMAb, its use for the delivery of radioisotopes either for imaging or for therapy purpose is safer than the MAb. Since the bsMAb can trap the radioisotope at the specific organ of interest and thereby reducing the required dose and the systemic exposure of the radioisotope. This strategy had been tested by different groups of researchers (Chetanneau *et al*., 1994; Chatal *et al.,* 1995; Dillehay *et al*., 1995; Kranenborg *et al*., 1995; Schuhmacher *et al*., 1995 and Sharkey *et al.*, 2010). The bsMAbs are also used in drug delivery (Cao and Suresh, 2000; Wang *et al*., 2005). The bsMAbs are also employed for different cancer immunotherapy (Van Dijk *et al*., 1991; Azuma *et al*., 1992; Knuth *et al*., 1994; Canevari *et al*., 1995; De Gast *et al*., 1995; Ohta *et al*., 1995; Burckhart *et al*., 2010; Fournier *et al*., 2010).

1.6. Aim of the study

1.6.1. Point of care (POC) assay

One of the major challenges in the global health care system is to control the infectious diseases, which cause 9.5 million deaths annually (WHO online report on infectious disease). Early diagnosis of the disease will not only facilitate timely initiation of the treatment, but also will enhance better control. The best way to describe the term POC diagnosis is an assay that is - affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and delivered to those in need (ASSURED) (Mabey, 2004). The POC detection tests can play an

important role in the developed and even more critical role in the resource constrained developing countries. The POC assay, which does not require sophisticated instrument and lab facilities can enhance detection capabilities in the poor countries where procurement of high tech lab facilities and personnel are major challenges. Several other factors such as emergence of antibiotic resistant pathogen, cost and regulation for developing new drugs, hastened epidemic to pandemic transition of infectious disease due to extreme globalisation (as observed in the recent swine flu outbreak), HIV infection – all underscore the importance of POC assay in the global public health care system. A rapid POC screening of the infectious disease can enable the quarantine of migrants from high disease burden countries at different ports of entry and that, in turn, can enhance the control of disease spread.

In the absence of a proper POC diagnosis, most of the vital infectious diseases (like TB and malaria) are diagnosed on the basis of symptoms that are non specific and as a result improper drug treatment often get initiated. The improper use of antibiotics facilitates the emergence of the drug resistant pathogen (Todar's online textbook of bacteriology).

Due to enormous development in the field of genome sequencing and high-throughput screening of disease markers, the search for POC detection assays have gained huge momentum in the recent past.

1.6.2. Immunoassays for TB detection

The enzyme linked immunosorbent assay (ELISA) is a biochemical technique to assess the amount of antibody or antigen in any fluid. Immunoassays involving antigen –

antibody interaction have been widely employed for rapid detection of different disease (as discussed earlier). Different formats of immunoassays have been employed to detect TB like using polyclonal, monoclonal antibodies and combination of both. The development of an ideal immunoassay for detecting TB has several challenges. Firstly, the bacteria expresses different kinds of antigens during different stages of the disease (Gennaro 2000; Laal and Skeiky 2004; Davidow *et al*., 2005; Gennarro 2005) and hence the antibody response is heterologous. Secondly, Bacillus Calmette-Guérin (BCG) vaccination and prior exposure to non pathogenic mycobacteria also can give false signal (Gennaro 2000). Thirdly, HIV co-infection affects the antibody profile for the TB and causes disseminated TB. The last two problems can perhaps be solved by detecting the TB specific antigen from different body fluids. Several studies have been reported that have detected different antigens (Banchuin *et al.*, 1990; Araj *et al*., 1993; Wallis *et al.*, 1998; Bentley-Hibbert *et al*., 1999; Chanteau *et al*., 2000; Landowski *et al*., 2001; Sumi *et al*., 2002; Choudhry and Saxena 2002 and Patel *et al.,* 2010).

Several immunoassay studies have been reported in the literature for the diagnosis of TB but unfortunately there are problems regarding the specificity and sensitivity. In spite of this fact, search is still going on to discover a suitable immunoassay for TB because of its intrinsic simplicity and rapid turnover time (Cho 2007). The immunoassay is most probably the single type of assay that has been implemented with various novel formats by coupling it with all possible optical, electrical and mechanical transducers (Yager *et al*., 2008). The reason for this continuous effort is perhaps that the immunoassays have all the potential characteristics of being an ideal diagnostic assay as famously acronymed by ASSURED (Mabey, 2004).

1.6.3. Antigen selection

Like any other pathogens, the mycobacteria also express three different types of antigens like protein, lipid and carbohydrates. Earlier studies have been reported regarding the detection of either different antigens or their corresponding antibodies. Some of the important work is mentioned in Table 2. (Palma-Nicolás and Bocanegra-García 2007).

Table 1.2: Some of the important TB antigens (Adapted from Palma-Nicolás and Bocanegra-García 2007)**:**

Antigen type	Name of the Antigen	Reference
Protein Antigens	ESAT6	Silva *et al.,* 2003
	CFP10	Dillion *et al*., 2000
	30 kDa protein	Sada *et al.*, 1990
	38 kDa protein	Espitia *et al*., 1989
	Mtb48	Lodes *et al.*, 2001
	Mtb81	Hendrickson *et al*., 2000
Lipid Antigens	Diacyl trehalose	Escamilla *et al.*, 1996
	Triacyl trehalose	Escamilla *et al.*, 1996
Carbohydrate Antigens	Lipoarabonomannan (LAM)	Chan *et al*., 2000
	Arabinomannan (AM)	Miller *et al*., 1983

Lipoarabinomannan (LAM) is an important, sero-dominant non protein antigen that constitutes almost 40% of the mycobacterium cell wall (Abebe *et al*., 2007). The LAM is primarily composed of carbohydrate (57%) and rest is lipid soluble material (29%), ninhydrin-reactive material (13.4%) and phosphate (0.68%) (Hunter *et al*., 1986). The carbohydrate portion is made up of two different sugars – arabinose and mannose – and their relative proportion in LAM is 1**:**0.64 respectively (Hunter *et al*., 1986). The molecule is basically made up of a mannose backbone with arabinose side chains and some of this side chain is further end capped with mannose. Initially it was thought that the extent of this mannose end capping dictates the virulence of the strain but later findings did not support this (Prinzis *et al.*, 1993). The bacteria are proposed to secrete the LAM antigen in order to manipulate the host immune system (Strohmeier and Fenton, 1999; Jozefowski *et al.*, 2008).

Previous studies have tried to detect LAM antigen and anti- LAM antibody from serum (Sada *et al.*, 1992), sputum (Cho *et al*., 1990; Pereira Arias-Bouda *et al*., 2000;), urine (Hamasur *et al*., 2001; Tessema *et al*., 2001; Tessema *et al*., 2002a; Tessema *et al*., 2002b; Boehme *et al*., 2005;) and cerebrospinal fluid (Patel *et al.,* 2010) sample of TB infected patients. The presence of LAM in urine is particularly helpful for its detection in the HIV co-infected population as well as in the extra pulmonary TB.

Based on the above discussion we have decided to design an assay that can detect LAM from the TB infected patients. Another reason for selecting carbohydrate antigen was that due to higher rate of emergence of MDR and XDR -TB, the bacteria is modifying the protein expression at a faster rate compared to that of the carbohydrate expression.

1.6.4. Synthetic carbohydrate antigen

In this study, we have decided to design an assay to detect LAM from the test samples. We selected the CS-35 MAb, which was raised against the leprosy LAM and also found to bind the LAM from the TB bacterium (Hunter *et al*., 1986), in order to develop the required the TB specific paratope on the bsMAb. For the initial design of the assay, we choose to work with a chemically synthesised hexasaccharide epitope of the LAM (Gadikota *et al.,* 2003) that was found to bind CS-35 with fairly good affinity (Rademacher *et al*., 2007). The reasons for working with the synthetic antigen were as follows:

- Biohazard free synthesis process
- Uniform and contamination free end product
- Better yield compared to the bacterial system
- Cost effective compared to the bacterial system
- Used only to design the assay not to raise the MAb (as mentioned before, CS-35 was raised against the bacterial LAM)

1.7. Objective & Hypothesis

The objective of the present study is to develop an immunoassay that will detect the TB at the POC level. As discussed before, in spite of having different tests for detecting TB, none of them can serve as a POC assay. That is why the development of new diagnostics for TB has been emphasized as one of the six strategies in the WHO recommendations to combat the

spread of the disease (Stop TB partnership website). The global market of the TB diagnosis is shown in Fig.1.6. The figure showed that almost 68% of the diagnostic market was dominated by the established market economies (EME) which include North America, Europe, Japan, Australia/New Zealand, although the high burden countries belong to the rest of the world (ROW) (TDR publication, 2006A). This discrepancy perhaps underscores the impact of a POC assay having the ASSURED characteristics (Mabey, 2004). We believe the immunoassay employing the bsMAb can serve the purpose.

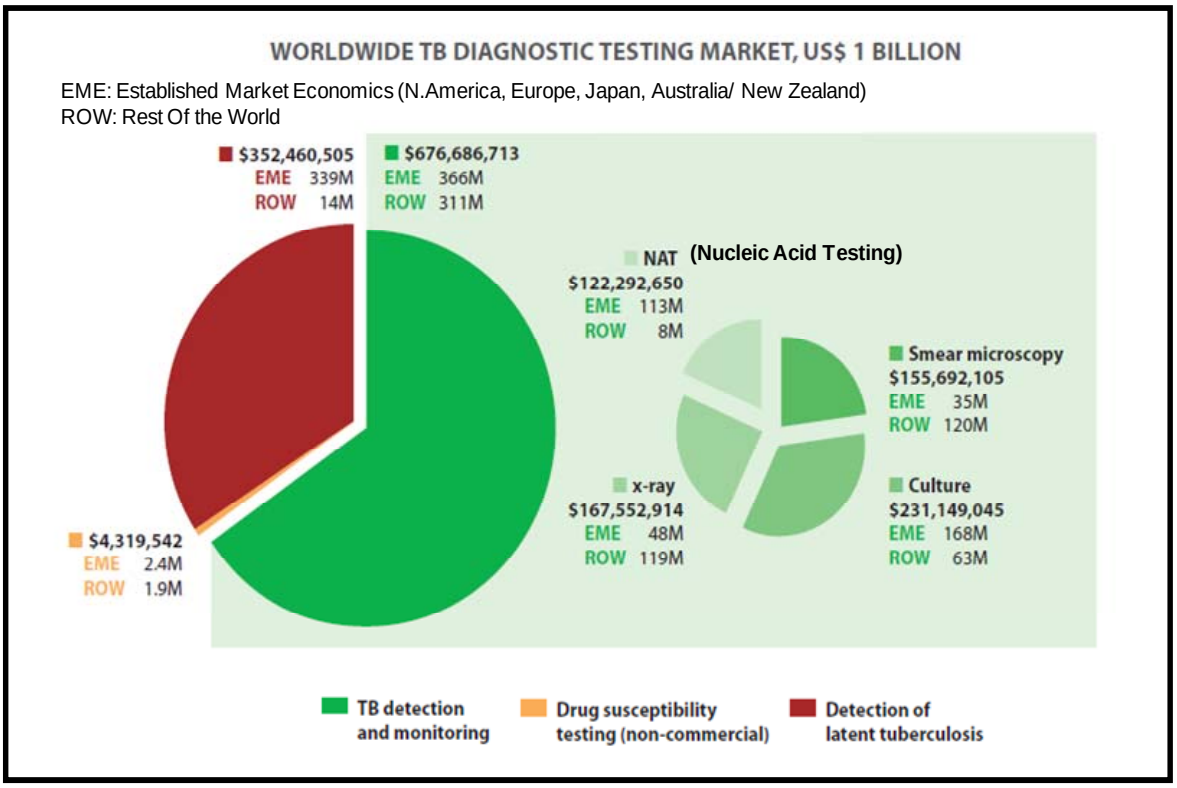


Fig. 1.6. The global market for total TB diagnostic testing. The data were collected between 2003-2004. The Figure was adapted from http://www.who.int/tdrold/publications/publications/pdf/tbdi/tbdi_chap3.pdf

The hypothesis of the study are as follows:

- The bispecific monoclonal antibody (bsMAb) developed by fusing CS-35 (secreted antibody against LAM) and YP4 (secreted antibody against the enzyme HRPO) will be able to detect the TB specific antigen LAM at the point of care level.

- The sensitivity of the immunoassay will be better in the bsMAb format than that of the chemically conjugated MAb (biotin labelled).

- The designed assay will be able to detect TB from clinical samples by detecting the antigen LAM.

1.8. Research plan

To achieve the aforementioned objective, the study was planned as follows:

- Expansion of the CS-35 MAb
- Checking the activity of the MAb
- Purification of CS-35
- Checking the extent of purity, activity and specificity of the MAb
- Biotin labelling of the pure CS-35 MAb
- Isolating the two fusion cell lines (CS-35 & YP4) from their exponential growth phase
- Somatic fusion of the above mentioned cell lines

- Screening and cloning of the bsMAb cell lines to get a stable cell line
- Expansion of the positive clones and purification of the final bsMAb clone
- Optimization of each of the assay parameters in a microtitre plate
- Checking the antigen detection limit
- Selection of a suitable swab
- Designing the immunoswab assay
- Comparison of the assay in bsMAb and biotin labelled MAb format
- Checking the ability of the designed assay to detect the antigen spiked in different matrices
- Checking the antigen detection limit in each matrix
- Checking the specificity of the assay
- Evaluating the assay with different bacterial antigens
- Evaluation of the assay with the stored clinical samples

Chapter II: Expansion, purification & biotin labelling of CS-35 MAb

2.1. Introduction:

Ever since the discovery of hybridoma technology by Kohler and Milstein, the biomedical research gained new momentum. The monoclonal antibody (MAb) became a handy tool to study different cell surface marker as well as different disease markers. In this study, the MAb producing CS-35 antibody was used to target the antigen LAM. The CS-35 was developed in mice by immunizing it with 100 µg of dry *Mycobacterium leprae* along with 50 µg of LAM, both formulated with incomplete Freund's adjuvant (Hunter et al., 1986). After the hybridoma fusion the clones were screened against LAM (Hunter et al., 1986).

This CS-35 was used in this study as the MAb and also as a fusion partner for generating the bsMAb having dual specificity towards the LAM and the enzyme HRPO. Extensive epitope mapping study suggested that CS-35 efficiently bind to a hexasaccharide epitope, composed of six arabinose sugars (Rademacher et al., 2007). As mentioned in the earlier chapter that the ratio of arabinose : mannose on LAM was found to be 1 : 0.64 (Hunter et al., 1986) so the hexasaccharide epitope was abundantly present on LAM and thereby justify the feasibility of designing a homosandwich ELISA based assay that would detect TB from different samples.

Due to the ease of conjugating MAbs with different chemicals, like biotin or different enzymes, CS-35 was also conjugated with biotin to check the performance of MAb based assay (Figure 4.1, format A).

2.2. Materials and Methods

2.2.1. Materials:

The cell culture media RPMI and Penicillin-streptomycin-glutamine (PSG) were purchased from Gibco (New York, USA). Fetal bovine serum (FBS) was bought from PAA laboratories (Pasching, Austria). Goat anti-mouse IgG conjugated with horseradish peroxidase (GAM-HRPO), bovine serum albumin (BSA), Protein G-agarose and long chain sulfosuccinimidyl NHS biotin were obtained from Sigma chemicals (St.Louis, USA). Streptavidin tagged HRPO (St-HRPO) was bought from BD Biosciences (California, USA). Tetramethyl benzidine (TMB) substrate was purchased from BioFx Laboratory (North Carolina, USA), while stabilised TMB substrate (for use in dot blot assay) was bought from Promega Corporation (California, USA). For Western blot, the hybond-ECL nitrocellulose membrane was procured from Amersham Biosciences, Germany and the chemiluminescence detection kit was procured from GE Healthcare (USA). For protein purification, Biologic Duoflow system (Bio-Rad, USA) was used. The non-sterile flat bottom NUNC maxisorp 96-well ELISA plates were purchased from VWR (Ontario, Canada). All plastic supplies and dialysis bags were purchased from Fisher Scientific (Houston, USA).

The monoclonal cell line producing CS-35 was obtained from Dr. J. S. Spencer, Departments of Microbiology, Immunology, and Pathology, Colorado State University, USA. The antigen BSA conjugated hexasaccharide epitope (BSA-Hex) of the LAM (Gadikota *et al.,* 2003) was obtained from Dr. T. L. Lowary's lab, Department of Chemistry, University of Alberta.

2.2.2. Expansion of CS-35 monoclonal antibody

After procuring the cell bank from the liquid nitrogen tank, it was thawed in RPMI media supplemented with 5% FBS and 1% PSG (in subsequent part of the thesis, this media will be referred to as 5% cell culture media) and centrifuged at 500g for 10 min. The supernatant was discarded and the pellet was suspended in the 5% cell culture media and cultured in 75 cm^2 sterile tissue culture flask at 37 °C with 5% CO_2 in the incubator under sterile conditions. The cell growth was monitored under microscope. Once the cells attained good viable condition, the culture was split into two flasks. The cell supernatant from one flask was transferred to a 50 ml centrifuged tube and spun down at 500g for 10 min. The other flask was kept in continuous culture with regular top-up with fresh media. The supernatant from the centrifuged tube was discarded and the pellet was suspended in the freezing mixture (10% Dimethyl sulfoxide in FBS) at a concentration of 1 X 10^6 cell/ml. One millilitre of this cell suspension was transferred in cryo vials and stored at -80°C for 2-3 days and finally transferred to the liquid nitrogen tank for future use.

2.2.3. Indirect ELISA

The activity of the CS-35 cell line was checked by adopting the indirect ELISA technique. The non-sterile microtitre plate was coated with 100 µl of the BSA-Hex antigen (8 µg/ml) in coating buffer (Na_2CO_3/$NaHCO_3$, pH 9.6) at 4°C overnight. The microtitre plate was then washed three times with phosphate buffer saline containing 0.05% tween-20 (PBST; pH 7.4) and the unoccupied binding sites were blocked using 2% BSA for 3 hours at 37°C. After washing three times with phosphate buffer saline containing 0.05% tween-20 (PBST; pH 7.4),

the cell supernatant (100 µl) was added and incubated for 2 hours at room temperature (RT). Subsequent to washing three times with PBST, goat anti-mouse IgG-HRPO (1:1000 dilution) was added and the plate was incubated for 30 minutes at RT. Finally, the plate was washed at least five times with PBST and finally TMB substrate was added. The developed blue color was read at 650 nm using the microplate reader.

2.2.4. Purification of CS-35 antibodies

CS-35 cell line was expanded in a hyper flask for large scale production of the protein and after 7-10 days the supernatant was harvested and centrifuged at 1000g for 20 minutes to remove cell debris. The supernatant was then purified using a Protein G-agarose column according to published protocol (Shahhosseini et al, 2006). Briefly, 1 litre of cell culture supernatant was passed through Protein G column using the Bio-Rad duo flow system and the unbound fractions were washed off using phosphate buffer saline (PBS; pH 7.4) and subsequently the bound protein was eluted using glycine (0.1M, pH 2.3). To reduce the denaturation of the protein at acidic pH, the eluted fractions were neutralized immediately using tris buffer (1M, pH 9.0) and dialysed in PBS overnight at 4°C with a minimum of three buffer changes. A small aliquot of each of original supernatant, unbound and elution fraction was collected for doing the successive characterisation of the protein.

2.2.5. Characterisation of CS-35 antibodies

2.2.5.1. Bio-Rad Protein assay:

The concentration of the pure protein was estimated by the Bio-Rad assay. This assay is based on the Bradford method (Bradford, 1976). The Bradford reagent forms a colored compound with the protein, the intensity of which can be read out using the microtitre plate. The intensity of the color depends on the concentration of the protein present in the sample. Then the concentration of the protein in the test sample can be estimated by comparing the absorbance value of the test sample with that of the known standards.

The assay was performed following the manufacture's protocol. Briefly, the dye solution was prepared by diluting the dye 5 times with water. Next, different standard solutions of BSA were prepared in the concentration range of 0 to 1 mg/ml (0, 0.1, 0.2, 0.4,.....1.0 mg/ml). Next, 10 µl each of standard and sample, in duplicate, was added separately in the microtitre plate and mixed with 190 µl of the diluted dye solution and incubated for 10 min at RT. The absorbance was then measured at 595 nm using the plate reader. The data were processed and the standard curve was constructed using the MS Excel software.

2.2.5.2. SDS-PAGE of the pure CS-35 antibody:

The extent of purification of the protein CS-35 was checked by running the Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following the published protocol (Laemmli, 1970). Briefly, a 12% resolving gel was prepared and allowed to polymerize. After that a stacking gel was prepared and poured over the previous one along with the lane making comb and the gel was allowed to set. Then the comb was removed and

10 µl of the reduced sample (protein sample was mixed with reducing dye and heated at 95°C for 15 minutes) was loaded in the gel along with the standard molecular marker in a separate lane. Then the gel was run at 90 V and with 35mA current for 1-1.5 hours (till the dye crossed the gel). Next the gel was immersed overnight in the staining buffer and finally in the destaining buffer for 2 hr.

2.2.5.3. Western blot of CS-35 antibody:

The extent of purification and activity of the pure MAb was examined by SDS-PAGE and Western blotting following the published protocol (Shahhosseini et al, 2006). Briefly, BSA-Hex and BSA were subjected to SDS-PAGE on two identical 12% gels and then one of the gels was stained as before while the separated proteins on the other gel was transferred onto a nitrocellulose membrane using the electro-blotting device. The membrane was then blocked with 5% skim milk for 2 hrs at RT, washed three times with PBST and subsequently incubated with 2 µg/ml of CS-35 pure protein for 1 hr. After washing, goat anti mouse IgG-HRPO (1:5000 dilution) was added to the membrane and incubated for 30 min. After washing, the chemiluminescent detection was performed using the kit.

2.2.6. Biotin labelling of CS-35

Biotinylation of CS-35 MAb was performed using previously published protocol (Shahhosseini et al, 2007). Briefly, 1 mg of the pure MAb was incubated with biotinamido hexanoic acid-3-sulfo-N-hydroxysuccinimide ester (long arm biotin; 20 µl of 3mg/ml) for 1

hour at RT. The reaction was stopped by the adding 10 µl of 100 µg/ml of glycine and the resultant protein was dialysed overnight in PBS to get rid of the unbound biotin.

2.2.6.7. Dot blot assay

The biotnylation of the protein was confirmed by doing a dot blot assay. A 10 µl dot of the labelled protein was made on the nitrocellulose membrane and incubated for 10-15 min. Then the membrane was blocked with 5% skim milk for 2 hrs at RT. After performing 3 times washing with PBST, the membrane was incubated with the conjugate, which was streptavidin tagged HRPO (1:10,000), for 30 mins at RT and after 5 times washing with PBST, TMB substrate was added. The control dot was made with the unlabelled protein.

2.3. Results

2.3.1. Activity of CS-35 antibody

The activity of the CS-35 cell line was checked by doing the indirect ELISA using the BSA-Hex antigen. The hexasaccharide (composed of arabinose sugar) of the conjugate was synthesised chemically and it is abundantly available in the mycobacterial LAM (lipoarabinomannan) against which the CS-35 was raised (Hunter *et al*, 1986). A steady increase in the absorbance value was observed with the successive increase in the coating antigen concentration. The control of the study was BSA where the absorbance value was almost negligible.

After plotting the absorbance value with the corresponding coating concentration, a steady rise in the absorbance value was observed with successive increase in the coating antigen (BSA-Hex) concentration and that was followed by a plateau region which ultimately merged to a straight line parallel to the concentration axis. The point of transition between steady rise to slow rise (above which the binding attained almost saturation), 4-8µg/ml was the range selected as the coating concentration for the antigen for all the subsequent indirect ELISA. The result was shown in Fig 2.1.

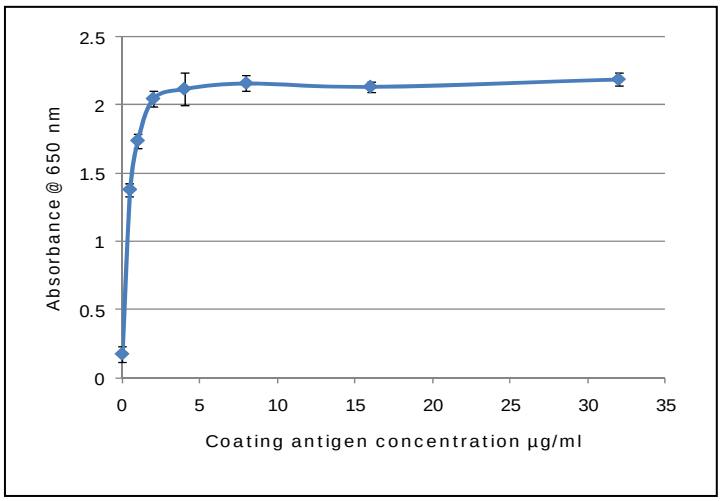


Figure 2.1: Optimization of coating antigen concentration.

2.3.2. Purification of CS-35

The purification was done using the principle of affinity chromatography. The ProteinG agarose was used, since the protein is of IgG isotype. The supernatant was loaded in the column and the unbound fraction was collected by washing with PBS. A sharp rise in the absorbance value was observed when the elution started at acidic pH. The purification was done using Biologic Duoflow system (Bio-Rad, USA). The purification profile was shown in Fig 2.2.

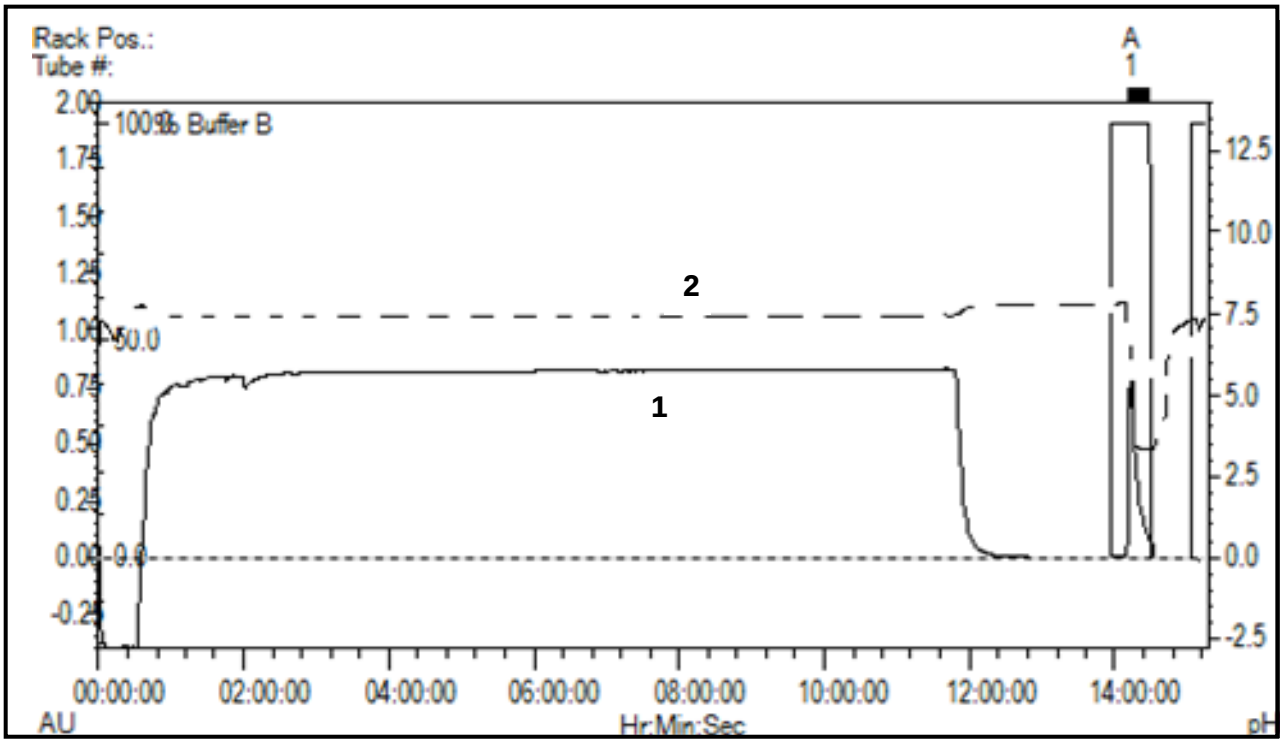


Fig 2.2. The purification profile of CS-35, as obtained from Bio-Rad duoflow system. The line marked '1' shows the protein absorbance value (absorbance scale was at left side in terms of AU) and the line marked '2' shows the pH profile (pH scale was at right side). At pH 2.5, the elution occurred which was represented by the steep rise in the absorbance line.

2.3.3. Characterisation of CS-35

2.3.3.1. Protein content and activity of the pure CS-35:

The initial fraction, unbound fraction and eluted fraction were analysed for their protein content and activity. In terms of protein content, assessed by the Bradford protein assay, the original supernatant showed maximum peak followed by the unbound fraction and lastly the eluted fraction. In terms of activity, assessed by indirect ELISA, the highest peak was obtained in the eluted fraction followed by the supernatant and finally the unbound protein which has almost negligible activity.

Since, the supernatant contain all the proteins, including IgG, it showed the highest protein content with considerable activity against the BSA-Hex antigen and followed by the unbound portion which contained all the same proteins except the IgG fraction, which explains the negligible ELISA activity. Since the eluted fraction had only the IgG fraction, it showed minimum total protein but maximum activity. At the end of the purification process, 4.9 mg of protein (0.55 mg/ml) was obtained. The total protein and the activity profile are shown in Fig.2.3.

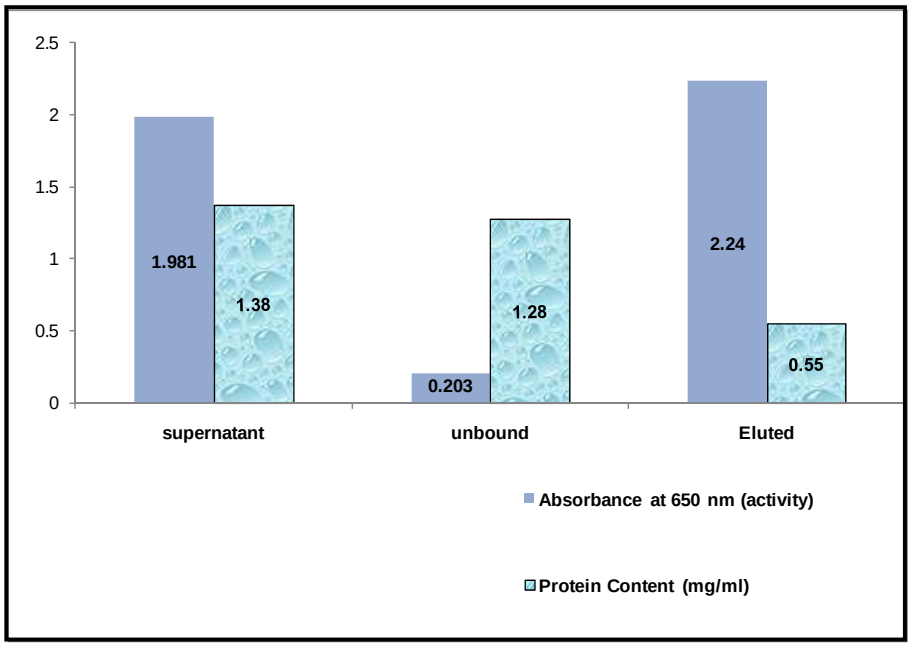


Fig 2.3. The analysis of different fractions obtained during purification of CS-35. The activity was expressed in terms of absorbance value and the protein content is expressed in terms mg/ml.

2.3.3.2. SDS-PAGE & Western blot analysis:

After staining the gel, two bands were observed around 27 kDa and one band was at around 50 kDa which correspond to the two light chains and one heavy chain of the IgG antibody respectively. The result is shown in Fig 2.4.

In the western blot analysis, distinct binding was observed in the lane containing the BSA-Hex antigen but no binding was observed in the lane containing only BSA. The result established the specific activity of pure CS-35 protein towards the hexasaccharide moiety but not towards the carrier protein, which is BSA. The result is shown in Fig 2.5.

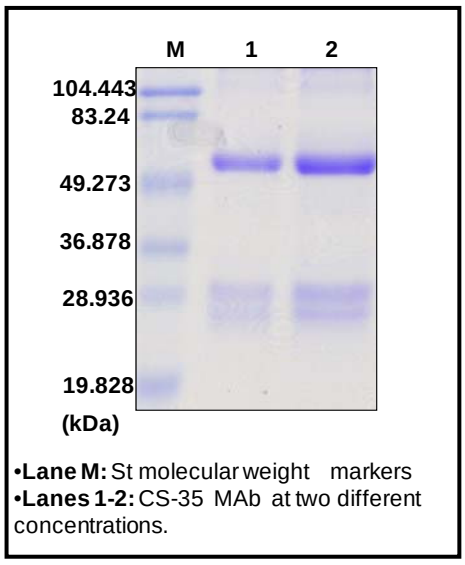


Fig 2.4. The SDS-PAGE analysis of CS-35. Two bands are obtained, one at around 50 kDa (heavy chain) and the other at around 25 kDa (light chian).

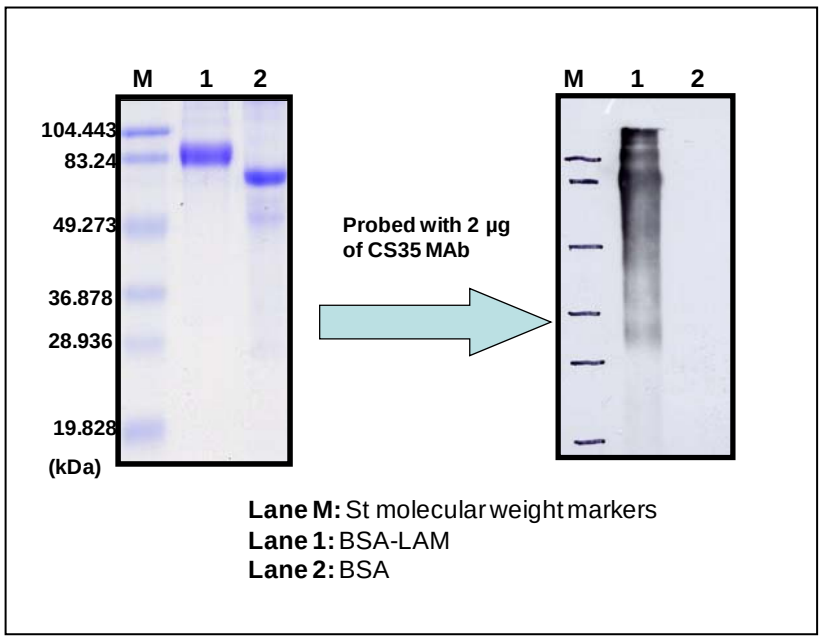


Fig 2.5. The Western blot analysis of CS-35. The dyed gel is shown in left side and the corresponding blot was shown at right. No band in the blot is observed in the lane containing BSA.

2.3.4. Biotinylation of CS-35

The biotinylation of the CS-35 was confirmed by the dot blot assay. After developing the color with the suitable substrate, blue dots were obtained in the row containing the biotin labelled protein but no dot was detected in the row containing the unlabelled CS-35. This could be explained by the fact that the conjugate St-HRPO can only bind to the biotin labelled CS-35 but not to the unlabelled one. The result is shown in Fig 2.6.

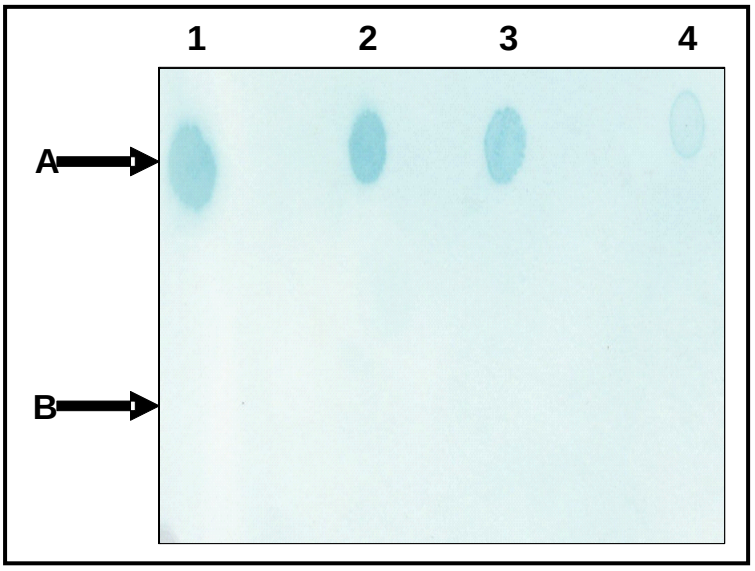


Fig 2.6. The dot blot of biotin labelled and unlabelled CS-35.

Row A: Bio-CS-35; **Row B:** CS-35;

Column 1- Neat (0.45 mg/ml); **Column 2**- 1:10; **Column 3**- 1:100; **Column 4**- 1:1000.

Chapter III: Production and characterisation of the bsMAb

3.1. Introduction

Ever since the invention of hybridoma technology by Kohler and Milestein in 1975 (Kohler et.al. 1975), it has found wide application in the field of medicine and biology. The bispecific monoclonal antibody (bsMAb) is a second generation monoclonal antibody that is being used extensively in therapeutics and diagnostics. The fusion of two hybridoma cell lines gives a quadroma cell line having two different paratopes (antigen binding site), which can be selected based upon the application of the bsMAb. Since the bsMAb generated here will be subsequently used to design a diagnostic assay for tuberculosis, one paratope of the quadroma was designed so that it could bind to a tuberculin antigen while the other paratope could bind to the enzyme HRPO (horseradish peroxidase).

The advantage of employing bsMAb over the conjugated MAb (either biotin or enzyme labelled) is the higher sensitivity of the former. Another advantage of using bsMAb is that during the purification stage bsMAb is purified as a bsMAb-HRPO complex which eliminates one extra step of adding the enzyme and subsequent washing steps. This helps to reduce the total time for the assay.

To develop the desired bsMAb, CS-35 and YP4 hybridoma cell lines were used. As discussed in the previous chapter, the CS-35 is a mouse hybridoma that specifically binds to the LAM antigen. On the other hand, YP4 is a rat hybridoma that binds to the enzyme HRPO. So the resultant fused quadroma cell line is expected to secret antibody having dual specificity towards LAM and HRPO. There are different techniques available for the bifunctional fusion (Cao et al.

1998). Here the somatic fusion technique was employed using two established (above mentioned) hybridomas. The technique involved various steps like – labelling the cell separately with two different flurophores, fusing the cells using chemicals, FACS sorting, screening of the positive clone, recloning and isolating a stable clone, expanding the stable clone and finally purifying and characterising the protein.

3.2. Materials & Methods

3.2.1. Materials

Cell culture media RPMI and Penicillin-streptomycin-glutamine (PSG) were purchased from Gibco (New York, USA). Fetal bovine serum (FBS) was purchased from PAA laboratories (Pasching, Austria). Goat anti-mouse IgG conjugated with horseradish peroxidase (GAM-HRPO), bovine serum albumin (BSA), polyethylene glycol (PEG) 1300-1600, fluorescein isothiacyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), HRPO Type IV, Protein G-agarose and m-amino phenyl boronic acid (m-APBA) agarose were purchased from Sigma chemicals (St. Louis, USA). Tetramethyl benzidine (TMB) substrate was purchased from BioFx Laboratory (North Carolina, USA). For western blot, the hybond-ECL nitrocellulose membrane was procured from Amersham Biosciences, Germany and the blot detection system was procured from GE Healthcare (USA). Non-sterile flat bottom NUNC maxisorp 96-well ELISA plates were purchased from VWR (Ontario, Canada). All plastic supplies and dialysis bags were purchased from Fisher Scientific (Houston, USA).

The fluorescence activated cell sorter - FACSAria (BD Biosciences, USA) - was accessed from department of Medical, Microbiology and Immunology, University of Alberta. The centrifuge model used was MSE Mistral 2000. For protein purification, we used Biologic Duoflow system (Bio-Rad, USA) and the ELISA absorbance was taken using Versa max microplate reader (Molecular devices, USA).

3.2.2. Cell lines for bifunctional fusion

CS-35: It is a mouse IgG hybridoma cell line raised against *M. leprae* LAM (Hunter *et al.,* 1986). This cell line was obtained from Dr. J. S. Spencer, Departments of Microbiology, Immunology, and Pathology, Colorado State University, USA.

YP4: It is an anti-HRPO rat IgG hybridoma cell line. The cell line was a kind gift from late Dr. C. Milstein, Medical Research Council for Molecular Biology, Cambridge, United Kingdom.

3.2.3. Bifunctional fusion

The fusion was performed following the published protocol (Das and Suresh, 2005; Tang et al., 2004 and Kreutz et al., 1998) with some modification. Two cell lines (CS-35 and YP4) were isolated from their logarithmic growth phase and washed separately with serum free RPMI media containing 1% PSG (SFM) for three times to get rid of the serum. Then CS-35 was suspended in 10 ml of SFM (pH 7.4) while the YP4 was suspended in SFM (pH 6.8). Then approximately 2.0 X 10^7 CS-35 cells were labelled with flurophore TRITC at a concentration of

2 µg/ml and similar number of YP4 cells were labelled with the other flurophore FITC at a concentration of 1 µg/ml. The cells were incubated for 30 min at 37°C. After that the cells were washed as before to get rid of the extra dye. After the third time washing, the cells were separately suspended in 10 ml media and then 5 ml (1 X 10^7 cell) of the labelled CS-35 was mixed with 5 ml of labelled YP4. The mixture was mixed well and centrifuged at 114g for 5 min and the supernatant was discarded.

To the cell pellets, 500 µl of 50% (w/v) polyethylene glycol (PEG) was added drop-wise with constant shaking at 37°C. Immediately after 2 min incubation, the toxic effect of the PEG was neutralised by adding 15 ml of RPMI media containing 10% of FBS and the mixture was centrifuged at 114g for 5 min and the pellet was dissolved in RPMI media containing 10% of FBS and the cells were incubated for 45 min at 37°C with 5% CO_2 incubator.

The cells were then sorted in FACS sorting machine and double positive cells were cultured in 96-well sterile tissue culture plate at a concentration of 1 cell/well. The plate was incubated at 37°C with 5% CO_2 for 1-2 weeks. The growth of the newly formed quadroma clones were checked under microscope and after 7-10 days the cell supernatant was tested using the bridge ELISA technique. The above mentioned fusion process is schematically shown in Fig 3.1.

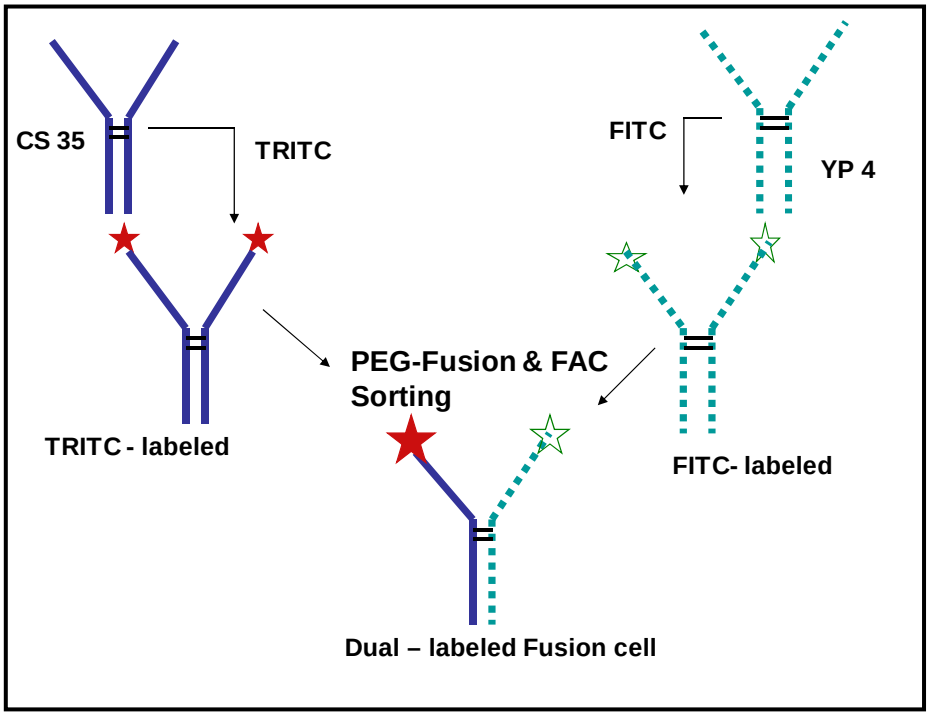
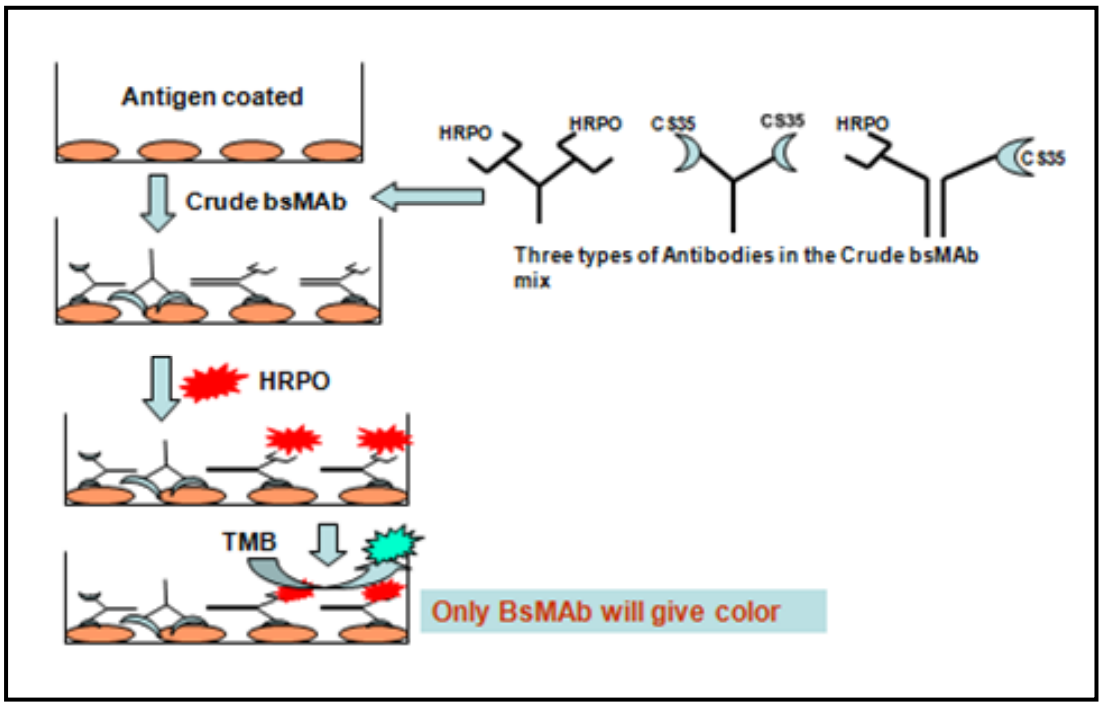


Fig. 3.1. The schematic representation of the bifunctional fusion.

3.2.4. Bridge ELISA

The screening of the quadroma cell line was done by exploiting the bridge ELISA technique. The technique is schematically shown in Fig 3.2. This technique was employed because the fused cell line is always a mixture of two parent hybridomas and the new quadroma (Suresh et al., 1986b). The bridge ELISA was performed following the published protocol with little modifications (Guttikonda et al., 2007). Briefly, the 96-well micro titre plates were coated with 100 µl of BSA-Hex (8 µg/ml) and incubated at 4°C for overnight. The plates were washed three times with PBST (PBS with 0.05% Tween-20) and the unoccupied sites were blocked with 2% bovine serum albumin (BSA) at 37°C for 3 hrs. After washing, 100 µl of serially diluted (neat, 1:10, 1:100) cell supernatant was added and incubated for 1 hour at room temperature (RT). The

plates were washed as before and 100 µl of HRPO (10 µg/ml) was added and incubated for 30 minutes at RT. The plates were washed well to get rid of any unbound HRPO, finally, 100 µl of TMB substrate was added and the developed colour was read at 650 nm using a microplate reader. The control was RPMI media only.



Fig,3.2. The schematic representation of the bridge ELISA.

3.2.5. Recloning of the cells by limiting dilution method

The positive clones were identified from the above test and expanded in 6-well culture plate. The cells were then recloned in 96-well culture plate at a concentration of 1 cell/well. The cell dilution was made by counting the viable cells using hemocytometer. The plate was

incubated at 37°C with 5% CO_2 for 1-2 weeks. The clones were screened as before by employing the bridge ELISA technique. This cloning and screening stage was repeated 5 times.

3.2.6. Purification of the bsMAb

After five cycles of cloning, the stable clone was allowed to expand in the hyper flask for protein purification. The supernatant was a mixture of antibodies secreted by three antibodies, two parent MAbs (CS-35 and YP4) and the third one is the fused bsMAb (Suresh et al., 1986b). The presence of CS-35 MAb would reduce the sensitivity of the assay in the subsequent steps of the assay. In order to isolate the CS-35 MAb from the bsMAb, a novel two stage affinity purification technique was employed (Bhatnagar et al., 2008). The first step isolated all IgG isotype antibodies from the non IgG proteins while the second step isolated the CS-35 protein from the other two (YP4 and the bsMAb). The first stage of the purification was same as that employed to purify CS-35 MAb (Chapter 2, article # 2.2.4). Briefly, the supernatant was passed through the ProteinG agarose column and the unbound fraction was washed with PBS and the pure IgG fraction was eluted with glycine (0.1M, pH 2.3). The entire stage purification operation was performed in Bio-Rad duo flow system. The eluted fraction was dialysed in PBS for overnight. The protein, thus obtained, contained only the antibodies of IgG isotype. Since all of CS-35, YP4 and bsMAb are of the IgG isotype so this fraction contains all the three.

The second stage of the purification involved m-amino phenyl boronic acid (APBA) agarose affinity column. The column was first incubated with HRPO (5 mg/ml) at 4°C for overnight and then unbound HRPO was removed by washing with potassium phosphate buffer (pH 7.4). The protein from the first stage was loaded on the column with a flow rate of 18 ml/hr. The unbound part (expected to contain CS-35) was washed with potassium phosphate buffer (pH

7.4) and finally the bound protein from the column was eluted with 0.1 M sorbitol in potassium phosphate buffer (pH 7.4). This second stage of purification is schematically shown in Figure 3.3. The elution fractions were analysed for its protein content and bispecific activity. The fractions showing maximum protein as well as activity were pooled together and dialysed in PBS overnight. The protein was then aliquoted into smaller fractions and stored at -20°C for future use.

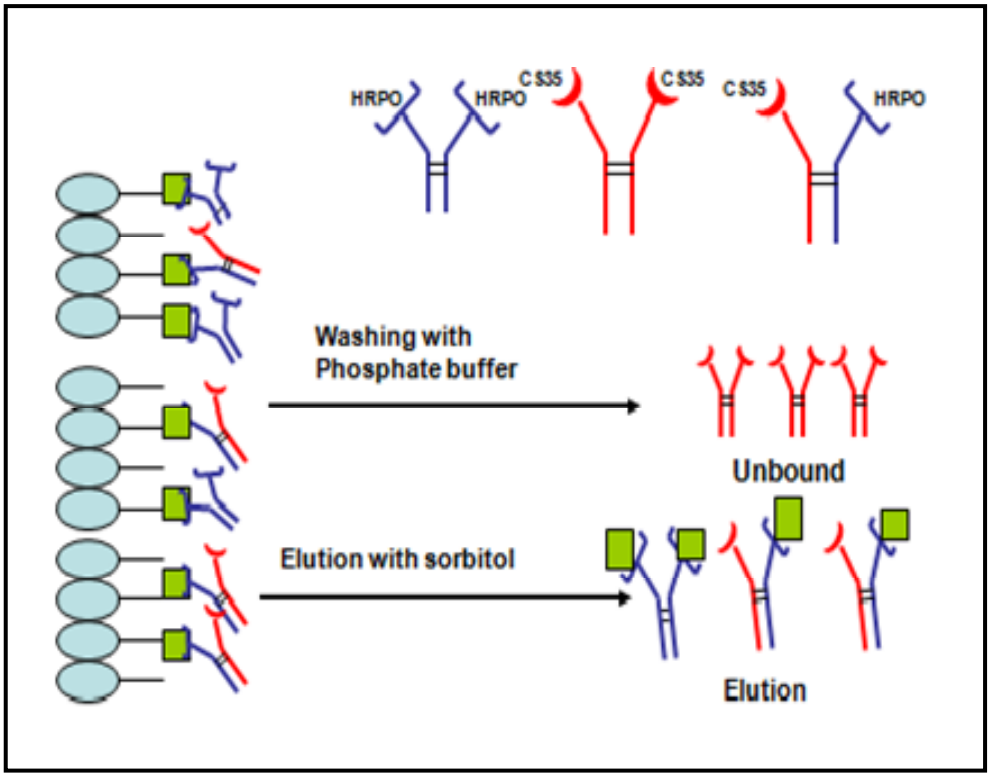


Fig.3.3. The schematic presentation of the second stage purification of bsMAb. The m-APBA column (represented blue circles) was saturated with HRPO (represented by green square) and then protein, after the first stage of purification, was passed through it.

3.2.7. Bradford and ELISA analysis of different fractions collected during purification

From the first stage of purification, initial supernatant, unbound and eluted fractions were collected and they were analysed for total protein content and activity by the Bradford assay and the bridge ELISA respectively. The Bradford assay was performed in exactly same way as mentioned previous chapter (see section 2.2.5.1).

The fractions from the first stage of purification were assessed by the bridge ELISA as mentioned above (see section 3.2.4) whereas the fractions after the second stage of purification was assessed by direct ELISA since HRPO had already been tagged with the bsMAb. In the latter case, the microtitre plate was first coated with 100 µl of 8 µg/ml of BSA-Hex antigen at 4°C for overnight. The unoccupied sites were blocked using 250 µl of 2% BSA and incubated at 37°C for 3 hrs. After washing with PBST for three times, 100 µl of different fractions (all the fractions were diluted according to their protein content) were added and incubated at RT for 45 min. Finally the plate was washed five times and 100 µl of TMB substrate was added and incubated for 10 min and the absorbance value was taken at 650 nm.

3.2.8. SDS-PAGE analysis of pure bsMAb

The SDS-PAGE analysis was performed according to the previously described protocol (see section 2.2.5.2). Briefly, a 12% resolving gel was prepared and allowed to polymerise. The stacking gel was prepared and poured over the previous one along with the lane making comb and gel was allowed to set. The comb was removed and 10 µl of the reduced sample (protein sample was mixed with reducing dye and heated at 95°C for 15 min) was loaded in the gel along

with the standard molecular marker in a separate lane. Then, the gel was run at 90 V and with 35 mA current for 1-1.5 hrs (till the dye crossed the gel). Next, the gel was immersed in the staining buffer for 2 hrs and finally in the destaining buffer overnight.

3.3. Results & Discussion:

3.3.1. Bifunctional fusion

Two cell lines, CS-35 and YP4, were isolated from their exponential growth phase and washed separately with serum free RPMI media to get rid of the serum (FBS). Next the 2 X 10^7 cells were counted and labelled separately with two different fluorophores. As mentioned earlier, CS-35 was labelled with TRITC and YP4 was labelled with FITC. After fusion, the cells were analysed by FAC sorting. The double positive cells were selected and seeded in the 96 well tissue culture plate at a concentration of 1 cell/well. According to the FAC sorting result, the percentage of double fluorescence was observed in 0.9% (Fig 3.4).

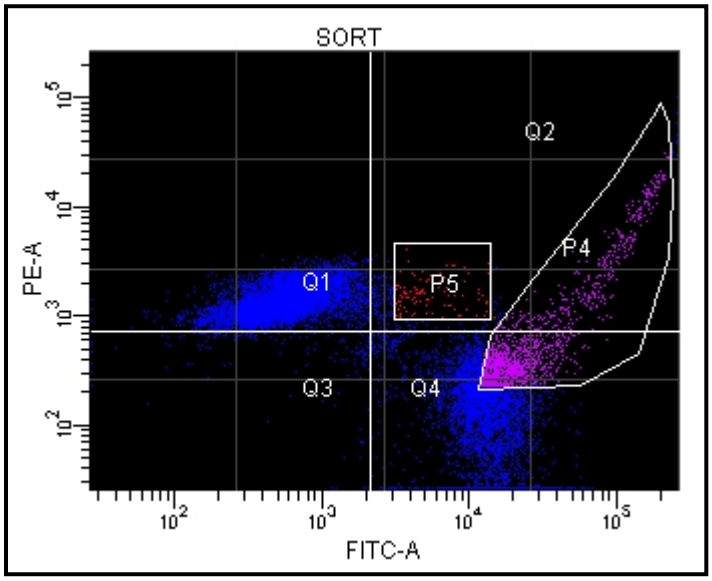


Fig.3.4. FAC sorting result. The FITC labelled YP4 is shown in Q4 and TRITC labelled CS-35 is in Q1. The dual labelled cells are shown in the square box marked P5.

3.3.2. Isolation of the stable clone

The sorted cells were cultured in 10% FBS media at 37°C with 5% CO_2 under aseptic conditions. The cell growth was monitored under microscope. Once the cells started to form a colony then the supernatant was tested by the bridge ELISA. The positive wells were selected where the absorbance value was at least 3 times blank. The positive primary clones selected after this was recloned by the limiting dilution method as mentioned before. After each cloning stage, cloning efficiency was calculated as follows:

Cloning efficiency (%) = (number of positive wells / number of wells tested on that stage) x 100

A gradual increase in the cloning efficiency was observed with the successive cloning stage and same trend in absorbance value was also observed. The data is shown in Fig 3.5. At the end of the fifth stage of cloning, the cloning efficiency reached above 85% and finally two stable clones of quadroma were isolated having dual affinity towards the TB antigen and the enzyme HRPO.

Those stable clones were expanded and cell banks were prepared following the standard protocol for cryo preservation.

The bsMAb fusion and bridge ELISA findings were in conformation with the earlier findings (Guttikonda *et al*. 2007, Kammila *et al.* 2008)

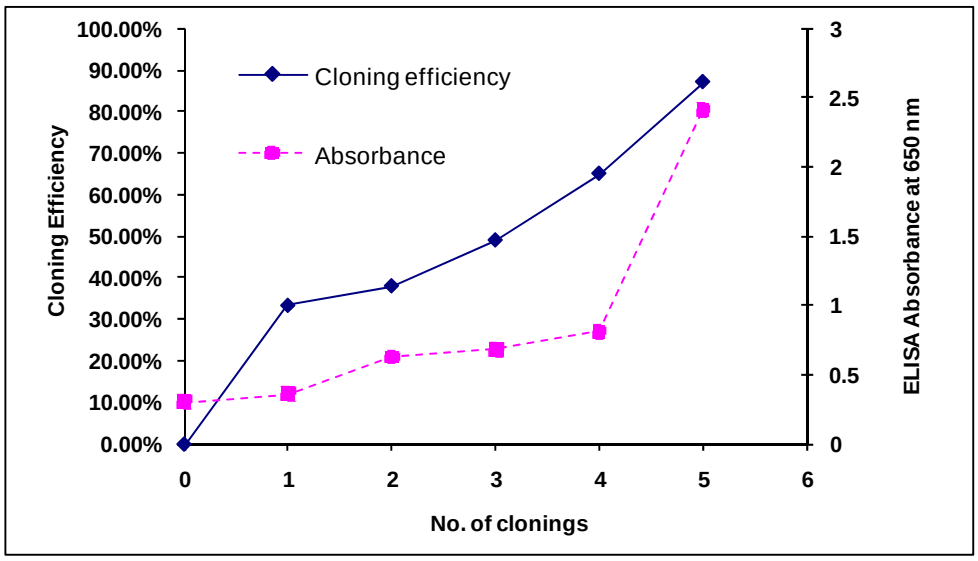


Fig.3.5. The relative enrichment of activity and cloning efficiency with successive cloning stage. The activity is depicted by the ELISA absorbance value.

3.3.3. Purification of bsMAb

The purification of bsMAb was achieved by two stage process. The first stage was exactly same as that performed with the CS-35 MAb. The initial supernatant, unbound fraction and the elution fraction were analysed for total protein content and bispecific activity by the Bradford analysis and the bridge ELISA respectively. In terms of protein content, the supernatant showed the maximum peak followed by the unbound and then the eluted fraction. However, in terms of activity, the eluted fraction was highest followed by supernatant and unbound.

The pure protein, obtained after the first stage of purification, was a mixture of three antibodies, the two parent hybridomas CS-35 and YP4 and the new quadroma. The presence of CS-35 in this pure protein would reduce the detection limit in the successive assay design. Since CS-35 will capture the TB antigen but cannot give any signal due to its inability to bind with HRPO and as a result the assay sensitivity will be reduced. In order to get rid of this problem, the

protein from the above stage was subsequently passed through the m-amino phenyl boronic acid (APBA) agarose column, which was previously being saturated with HRPO. Due to the presence of suitable paratope required to bind HRPO, the hybridoma YP4 and the quadroma would attach to the column and CS-35 would be washed out as unbound. Subsequent elution with the sorbitol would give the purified bsMAb and the YP4 both tagged with HRPO. The eluted fractions were analysed for protein content and activity. The fractions with maximum activity and protein were pooled and dialysed in PBS and the data is shown in Fig 3.6. The different fractions, collected at the end of the entire two stage purification process, were also analysed in terms of activity and IgG content and the result was shown in Fig 3.7. The similar type of purification profile was obtained by other researchers using the same protocol (Bhatnagar et al., 2008).

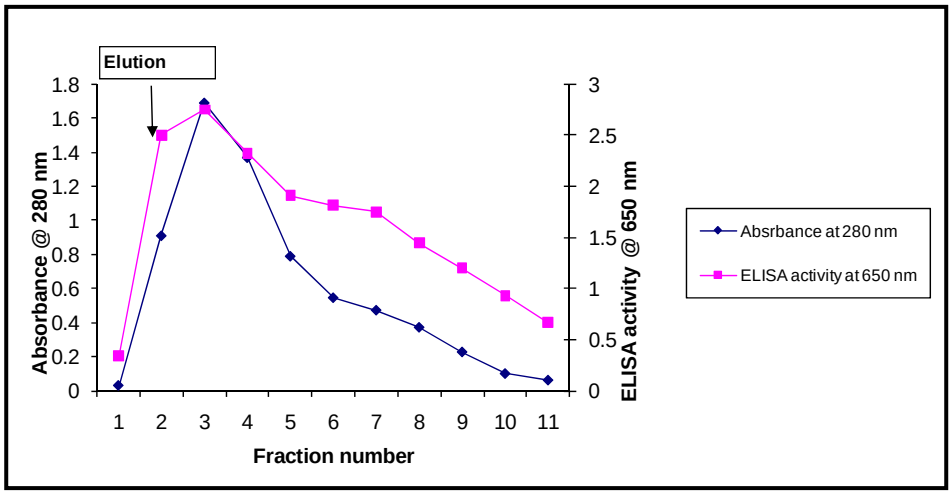


Fig.3.6. Analysis of different elution fractions from m-APBA column. The activity is shown in terms of ELISA absorbance and protein content is shown in terms of absorbance at 280 nm.

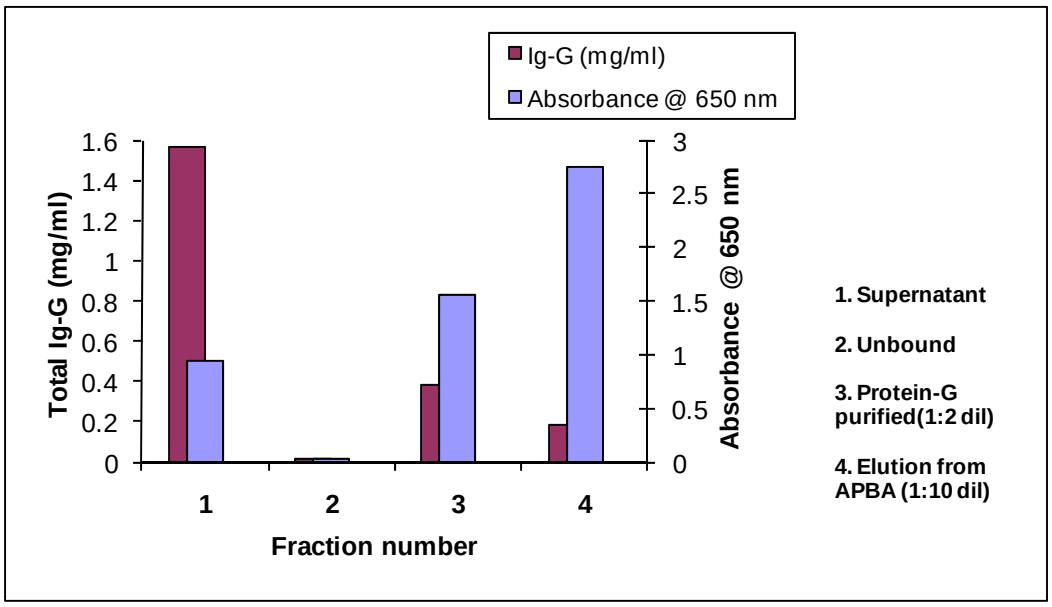


Fig.3.7. Analysis of different fractions obtained from two stage purification. The activity is shown in terms of ELISA absorbance and IgG content is shown in terms of mg/ml. The individual fractions were mentioned at the right hand side of the Figure.

3.3.4. SDS-PAGE analysis

The purified protein obtained after two stage of purification was analysed in SDS-PAGE to check the purity. Three distinct bands were obtained and after comparing with the standard molecular weight marker, the bands were found to be at around 25 kDa (corresponds to the light chain of IgG), 40 kDa (corresponds to the HRPO) and 50 kDa (corresponds to the heavy chain of IgG). The scanned photo of the gel is shown in Fig 3.8.

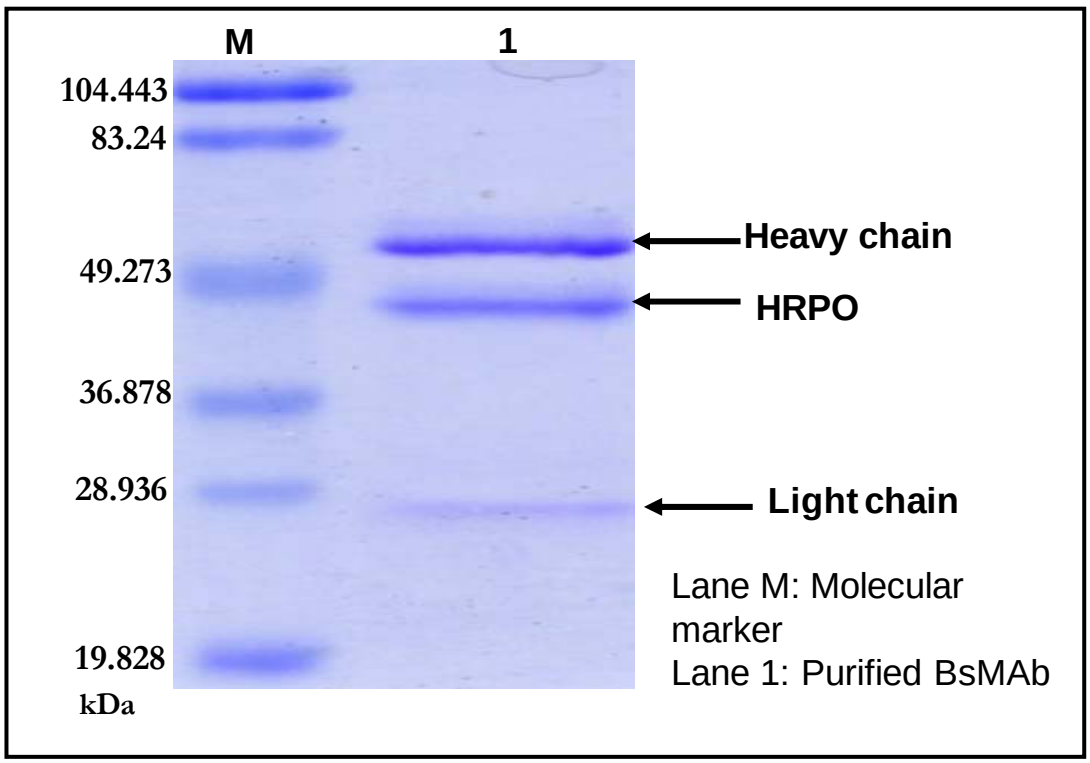


Fig 3.8. SDS-PAGE of the bsMAb. Three distinct bands are visible in the gel.

Chapter IV. Designing the immunoassay

4.1. Introduction

The sandwich ELISA format was used to design the assay. The idea of the assay is to coat the assay platform (either microtitre plate or swab) with a monoclonal antibody (MAb) CS-35 followed by the blocking of the unoccupied sites. The test sample containing the antigen was added and incubated. The detection antibody (either biotin labelled CS-35 or bsMAb) was added. The assay using the biotin labelled MAb, required another extra step of conjugate (st-HRPO) addition. However, this step is not required where the bsMAb was used as detection antibody since bsMAb already tagged with the enzyme HRPO. Finally the colour was developed with the substrate.

In order to design a good and sensitive assay, each parameters of the assay have to be optimized. The optimized conditions would not only enhance the sensitivity of the assay but would also prevent the unnecessary wastage of excess reagents and antibodies. To optimize the parameters, different sandwich ELISA assays were performed by varying one parameter at a time. Those optimized parameters were subsequently used to determine the sensitivity of the assay. The entire optimization and determination of sensitivity were first performed in the microtitre plate and then the parameters were proportionally transferred to the swab format.

Initially, we designed the assay to detect the antigen from the PBS buffer and later the antigen was spiked in various body fluids like rabbit serum, bovine urine and saline to mimic human serum, urine and sputum. The above mentioned matrices were chosen because

during the TB infection, the LAM antigen was found to be present in serum (Sada et al. 1992), urine (Boheme et al. 2005) and sputum (Arias-Bouda et al. 2000).

As mentioned in the first chapter, all throughout the work the assay was done using the synthetically generated hexasaccharide epitope of the TB antigen LAM (Gadikota et al., 2003). In order to make the hexasaccharide bind easily to the microtitre plate, it was conjugated with a carrier protein, BSA. There were certain advantages of using this synthetic antigen in the primary assay design over the natural bacterial counterpart. Firstly, the synthetic variety has uniform batch to batch composition compared to the bacterial counterpart. Secondly, the yield in the bacterial system is usually quite low. Finally, from the biosafety point, the generation of synthetic antigen does not require any specialised biosafety facility whereas the bacterial culture does. It is important to note that Mtb is a biosafety level 3 bacteria (Bloom B R. 1994). So it was comparatively easier (in terms of the uniformity of the end product), safer and cheaper to generate the synthetic variety.

At the end, the assay was evaluated using the bacterial LAM to check whether the assay could recognise the whole bacterial antigen or not.

4.2. Materials & Methods

4.2.1. Materials

The purified MAb, CS-35, and the biotin labelled CS-35 were processed in our lab as per the procedure described in Chapter 2. Streptavidin tagged HRPO (St-HRPO) was purchased from BD Biosciences (California, USA). Tetramethyl benzidine (TMB) substrate was purchased from BioFx Laboratory (North Carolina, USA. Different kinds of swabs were purchased as follows: nylon fibre swab from Micro Rheologics (Brescia, Italy); knitted polyester tipped swab from Puritan (Maine, USA); sterile cotton tipped swab from Pur-Wraps (Maine, USA); cotton tipped swab from Puritan (Maine, USA); calgiswab from Spectrum (California, USA). Non-sterile flat bottom NUNC maxisorp 96-well ELISA plates were purchased from VWR (Ontario, Canada). All plastic supplies and dialysis bags were purchased from Fisher Scientific (Houston, USA). The centrifuge model used was MSE Mistral 2000. For protein purification, we used a Biologic Duoflow system (Bio-Rad, USA) and the ELISA absorbance was taken using Versa max microplate reader (Molecular devices, USA).

Rabbit serum was obtained from Health Sciences Laboratory Animal Services (HSLAS), University of Alberta. Bovine urine was kindly arranged by Dr. Hoon H. Sunwoo, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta.

Native LAM from *M. tuberculosis* H37Rv strain was obtained from the Biodefense and Emerging Infections Research Resources Repository Manassas, Virginia, USA. The non-specific

antigen BSA-conjugated human blood group-A trisaccharide was a kind gift from Professor David R. Bundle, Department of Chemistry, University of Alberta, Canada.

4.2.2. Optimization of the assay parameters

4.2.2.1. Optimization of blocking buffer

The assay was first standardized in the microtitre plate and different blocking buffers were used to determine which would give the minimum background. To do so, the plate was first coated with 100 µl of CS-35 (6 µg/ml) and incubated at 4°C overnight. The plate was washed three times with phosphate buffer saline containing 0.05% tween-20 (PBST; pH 7.4). Then 200 µl of different blocking buffers - bovine serum albumin (BSA), gelatine, skim milk (SM), fetal bovine serum (FBS) and glycine - were used and the plate was incubated at same temperature and time. The different blocking buffers are shown in Table 4.1. After the incubation, the plates were washed. Four wells were blocked in each condition, in two of them 100 µl of BSA-Hex (10 ng/ml) antigen was added and in the rest PBS was added. The plate was incubated for 1 hr at room temperature (RT) and then it was washed with PBST buffer 3 times. Then 100 µl of bsMAb (4 µg/ml) was added and incubated for 45 min. After washing the plate five times with PBST, the TMB substrate was added. The developed colour was read at 650 nm.

Table 4.1. Different blocking buffers used in microtitre plate:

Blocking agents	**Incubation Temperature**	**Incubation Time**
3% BSA	37°C	3 hr
2.5% Gelatin	37°C	3 hr
2% BSA + 1% Gelatin	37°C	3 hr
5% Skim milk (SM)	37°C	3 hr
2% SM + 1% BSA + 1% Gelatin	37°C	3 hr
5% FBS	37°C	3 hr
2% FBS + 1% BSA + 1% Gelatin	37°C	3 hr
No blocking (PBS)	37°C	3 hr

4.2.2.2. Optimization of capture antibody (CAb)

Purified CS-35 MAb was employed as the capture antibody in the assay. A microtitre plate was coated overnight at 4°C with 100 µl of different concentrations of CS-35 MAb ranging from 0 to 16 µg/ml (0, 1, 2, 4...16) in triplicate. After washing, the blocking step was performed with 250 µl of 3% BSA in PBS at 37°C for 3 hours. The plate was washed 3 times with PBST and 100 µl of 5 ng/ml BSA-Hex antigen was added and incubated for 2 hours, and washed as before. Subsequently 4 µg/ml of biotin labelled antibody (DAb) was added and incubated for 1 hour and the plate was washed. To this St-HRPO (1: 5000 dilution) was added and incubated for 30 minutes. The plate was washed 3-5 times with PBST. Finally TMB was added and the developed colour was read at 650 nm using the microplate reader. The mean of three readings for each concentration was plotted against the corresponding concentration using Microsoft Excel.

4.2.2.3. Optimization of detection antibody (DAb)

The detection antibody concentration was optimized by using biotin labelled CS-35 MAb. A fixed concentration of CAb (8 µg/ml) was used to coat the plate and varied concentrations of DAb ranging from 0 to 16 µg/ml (0, 1, 2, 4...16) were used. The assay protocol and the concentration of the other parameters were same as described in section 4.2.2.2 and the data were similarly analyzed.

4.2.2.4. Optimization of the conjugate

Different dilutions of the conjugate St-HRPO (in PBS with 1% BSA) ranging from 1:4000 to 1:48000 (1: 4000, 1:8000.....1:48000) were used in the assay. The dilutions were made with using the PBS buffer. A control without any conjugate was also included. The concentrations of the other components such as CAb (8µg/ml), DAb (2µg/ml) and BSA-Hex antigen (5 ng/ml) were kept constant. The assay was performed as described in section 4.2.2.2 and the data were similarly analyzed.

4.2.3. Antigen detection in the microtitre plate

After optimizing different assay parameters, the sensitivity of the assay was determined by using the optimized conditions. The sensitivity of the assay was determined by measuring the lowest amount of the antigen that can be detected by the assay. Different concentrations of the BSA-Hex antigen ranging from 15 ng/ml to 0 (15, 7.5.....0) were used. The assay was performed in triplicate and repeated twice.

4.2.4. Evaluation of different immunoswab

Five different types of commercially available swabs were examined: nylon fibre swab, knitted polyester tipped swab, sterile cotton tipped swab, cotton tipped swab, and Calgiswab. The swabs were directly blocked by 300 µl of 5% BSA for 45 min at RT then the swabs were washed with PBST for three times and incubated with 100 µl of HRPO (10 µg/ml) for 20-30 mins. The swabs were washed for 5 times and TMB was added. The assay was performed to

select for swab(s) giving minimum background i.e. minimum color in absence of any signal (antigen).

4.2.5. Selection of the blocking buffer for swab

The next task was to identify the best blocking buffer for the swab. This step was somewhat similar to that was performed in the plate (see 4.2.2.1). The swabs were blocked using different blocking buffers such as 5% BSA, 5% FBS, 5% gelatin, 5% skim milk, and a commercially available blocking buffer (blocking buffer supplied with commercially available ELISA kit). All of the dilutions were made with PBS and two swabs were tested in each condition.

4.2.6. Immunoswab assay with biotin labelled MAb and bsMAb

Both the MAb- and the bsMAb-based assays were done in the immunoswab format. In both the format CS-35 was used as capture antibody. In the MAb based format, biotin labelled CS-35 was used as detection antibody and the enzyme (St-HRPO) was added separately. Whereas in the bsMAb based format, the detection antibody was bsMAb, complexed with HRPO. The schematical representation of two formats is shown in Fig 4.1.

First, the swabs were coated with CS-35 MAb (50 µL of 25 µg/ml in PBS) at RT for 30 minutes, dried for 5 minutes and fixed with 50 µL of 95% ethanol for 1 min. The swabs were then blocked with 5% DBSA in PBS at RT for 45 minutes and were washed five times using PBST with 1 minute incubation in each washing step. Washings were performed by a simple fill and aspiration method in a 1 ml Eppendrof tube. The washed swabs were incubated for 30 minutes with different concentrations of BSA-Hex antigen in PBS containing 1% DBSA.

Subsequently, the swabs were washed and incubated for 30 minutes with either bsMAb (50 µL of 10 µg/ml in PBS containing 1% DBSA) in the bsMAb-based assay or biotin labelled MAb (50 µL of 4 µg/ml PBS containing 1% DBSA) in the MAb-based assay. In the bsMAb-based assay, swabs were washed five times as mentioned above and finally 100 µL of TMB was added for colour development. On the other hand in the MAb-based assay, the swabs were incubated with St-HRPO (1:10000 dilution in PBS containing 1% DBSA) for 30 minutes and were washed before TMB was added. The endpoint in both formats was detected visually and compared with the blank swab that was treated as described above except that it was incubated in PBS instead of any antigen. Following colour development, the swabs were scanned using an Epson scanner.

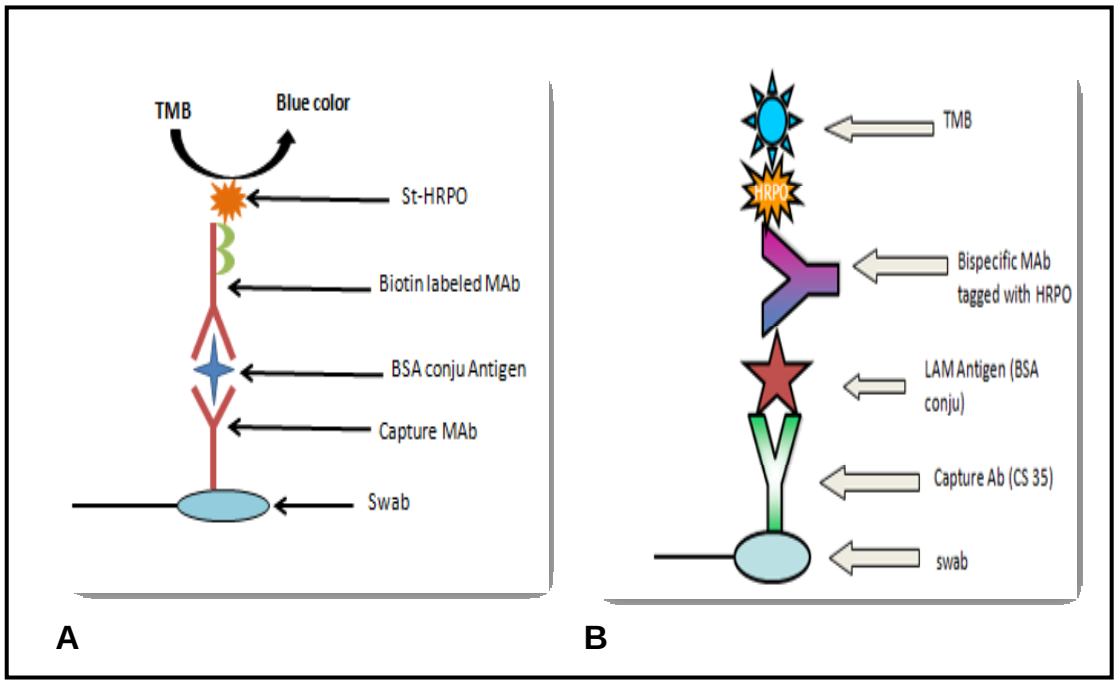


Fig.4.1. Two different sandwich ELISA formats. A: Biotin labelled MAb based; **B:** bsMAb based.

4.2.7. Antigen spiking in different matrices

After performing the assay in the PBS buffer, different matrices were chosen to spike the antigen. The matrices used were saline, rabbit serum and bovine urine to mimic the human sputum, serum and urine sample. After finalising the different matrices, the BSA-Hex antigen was spiked in them.

4.2.8. Antigen detection limit in different matrices

Next the immunoswab assay was performed on each matrix to determine the detection limit of the antigen. The assay protocol was same as described in section 4.2.6. Two swabs were tried at each concentration.

4.2.9. Evaluation of the assay with the bacterial antigen

After determining the detection limit with the synthetic antigen, the immunoswab assay was evaluated using the bacterial LAM. This bacterial LAM was obtained from the virulent strain of Mtb. The antigen was spiked in rabbit serum and the assay was performed as before. Due to limited availability of the bacterial antigen, the assay was only performed in the serum matrix.

4.2.10. Evaluation of the assay with different saccharides:

In order to check the specificity of the assay, two different saccharides were used since the target antigen LAM is a polysaccharide. The human blood group A trisaccharide (conjugated with BSA) and sucrose were used as non specific antigens along with the specific BSA-Hex antigen. The assay was performed by spiking the antigens in the PBS buffer.

4.2.11. Dot blot with different bacterial LAM and synthetic antigens:

In nitrocellulose membrane, 6 different dots were made with 6 different antigens – synthetic BSA conjugated hexaarabinofuranoside (BSA-Hex), the same hexaarabinofuranoside conjugated with tetanus toxoid (TT-Hex), bovine serum albumin (BSA), LAM from *Mycobacterium tuberculosis* (TB-LAM), *Mycobacterium leprae* (Lep-LAM) and *Mycobacterium smegmatis* (Smeg-LAM). The amount of antigen per dot was 1 µg for each of them. The dots were allowed to blot on the membrane for 5-10 min. Then the membrane was blocked with 5% skim milk for 45 min. Next it was washed with PBST 3 times and 3 ml of biotin labeled CS-35 (4 µg/ml) was added and incubated for 45 min. After washing, 3 ml of streptavidin-HRPO (1:10,000 dilution) was added and incubated for 20 min. The membrane was finally washed 5 times with PBST buffer and the color was developed using TMB substrate. After color development, the membrane was scanned in a scanner. The assay was also repeated with bsMAb in place of biotin labeled CS-35 and St-HRPO.

4.3. Results & Discussion:

4.3.1. Optimization of the assay parameters

4.3.1.1. Optimization of blocking buffer

To get maximum signal/noise (S/N) ratio, different blocking buffers were tested. Initially after coating, different blocking buffers (as mentioned in Table 4.1) were added and 4 wells were blocked under each condition. After blocking, the BSA-Hex antigen was added in two wells (signal) and only PBS was added in the remaining two wells (noise). After getting the absorbance value, the S/N ratio was calculated by dividing the corresponding absorbance values and they were plotted against the corresponding blocking condition. The result is shown in Fig 4.2. As evident from the figure, the blocking condition of 3% BSA at 37°C for 3 hrs worked best since it gave maximum S/N ratio. For the rest of the study, this blocking condition was used to block the unoccupied sites in the micro titre plate.

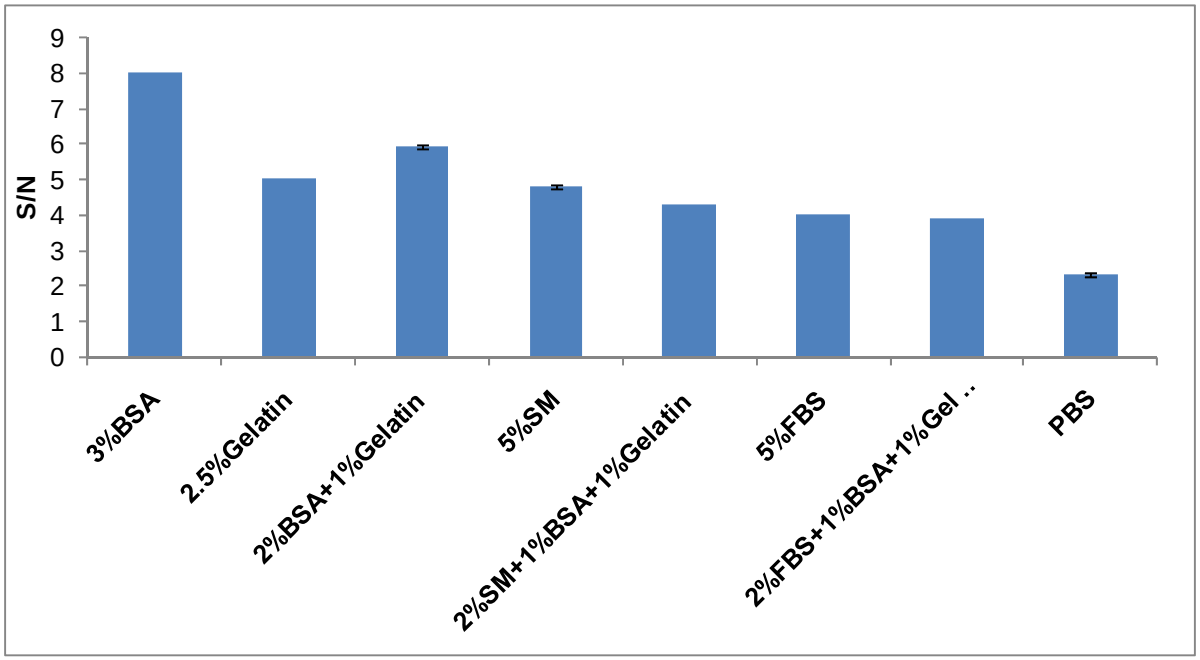


Fig 4.2. Optimization of different blocking conditions in micro titre plate. Signal/noise (S/N) ratio is plotted against the corresponding blocking conditions.

4.3.1.2. Optimization of capture antibody

Different concentrations of CS-35 were used to coat the microtitre plate. The concentration-range used was from 16 µg/ml to 0 (16, 8.....0), and each concentration was in triplicate. After getting the absorbance value at 650 nm, the data were analysed by Excel software. The average of the three readings was plotted against their respective concentration. Initially a steady increase in the absorbance value was noticed with the increase in the coating concentration and finally a saturation point was attained when the graph became almost parallel to the concentration axis. The result is shown in Fig 4.3. The point of transition between the sharp rise and slow rise was chosen as the optimized condition as binding saturation (almost negligible increase in absorbance value with subsequent increment in the concentration) was attained beyond that concentration. The optimized coating concentration was found to be 8 µg/ml.

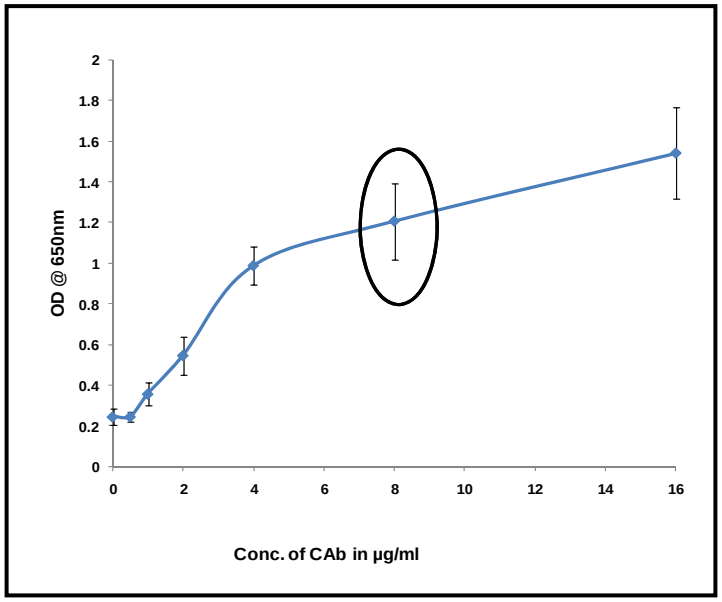


Fig.4.3. The optimization of capture antibody (CAb) concentration. The encircle point was chosen as optimized concentration.

4.3.1.3. Optimization of detection antibody

The optimized concentration of the detection antibody was determined similarly and the result is shown in Fig 4.4. The data analysis was performed in exactly the same way as mentioned above in 4.3.1.2. The optimized detection antibody concentration was found to be 4 µg/ml.

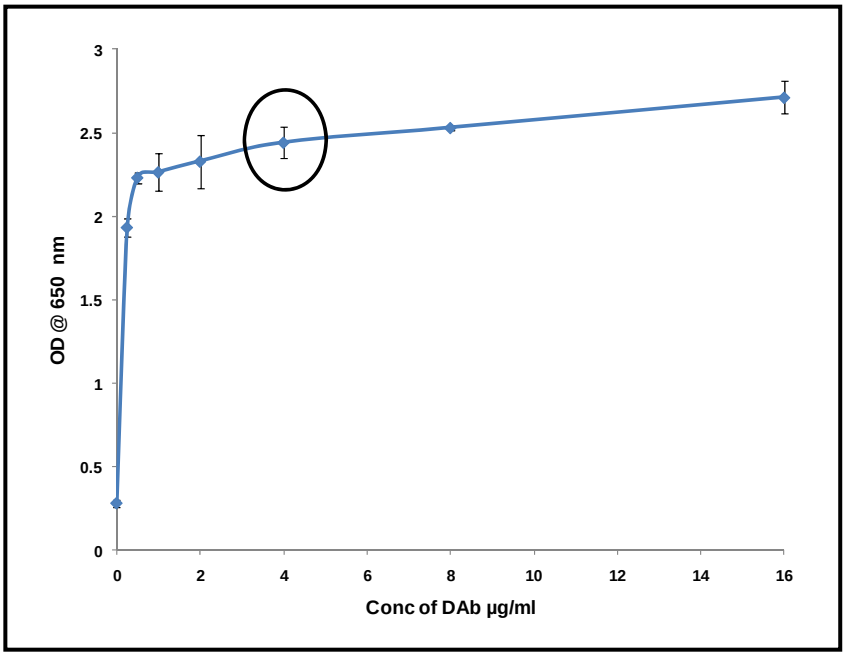


Fig.4.4. The optimization of detection antibody (DAb) concentration. The encircle point was chosen as optimized concentration.

4.3.1.4. Optimization of the conjugate

The assay, involving biotin labelled CS-35 as detection antibody, streptavidin tagged HRPO (st-HRPO) was used as the conjugate. The optimized dilution of the conjugate was determined similarly and the result is shown in Fig 4.5. The data analysis was performed in the exact same way as mentioned above in 4.3.1.2. The optimized dilution of the conjugate was found to be 1: 8,000.

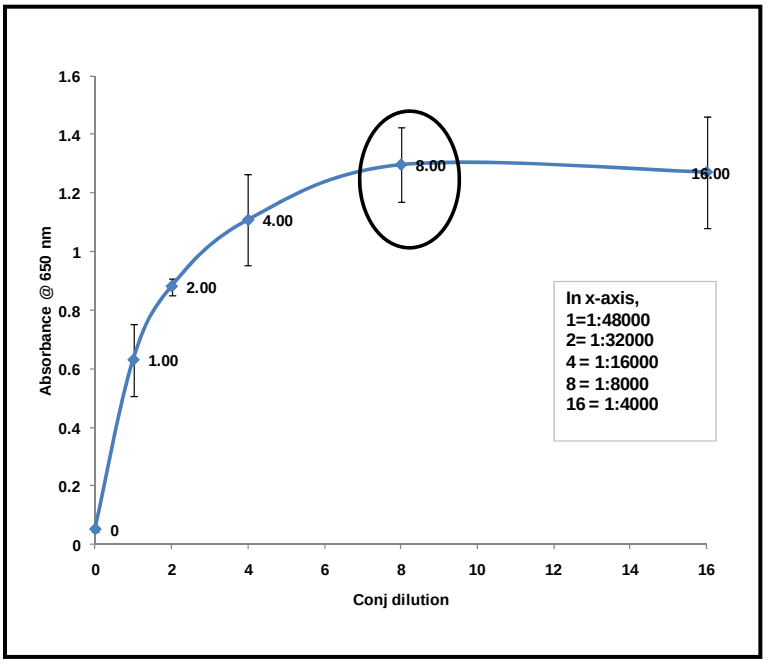


Fig. 4.5. The optimization of the St-HRPO dilution. The optimized condition is encircled.

4.3.2. Antigen detection limit in the micro titre plate

All the above mentioned optimized conditions were used to determine the lowest limit of antigen detection. Serial dilutions of antigen were used, starting from 15 ng/ml to 0.01 ng/ml. All the concentration was performed in triplicate. The control of the study was the diluting buffer (PBS) without any antigen. As before, the average of three readings was plotted against the

corresponding antigen concentration. The statistical significance of the each point was determined by using the student t-test and 0.234 ng/ml was found to be the limit of detection in the microtitre plate. The result was shown in Fig 4.6.

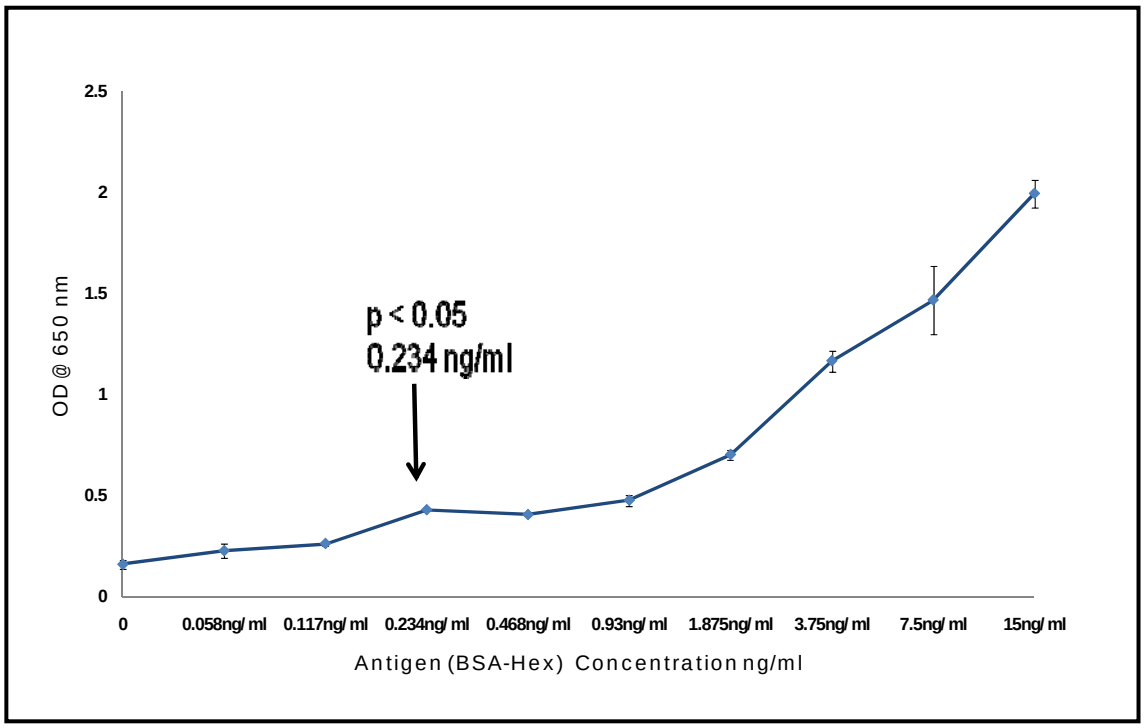


Fig.4.6. The antigen detection limit in the micro titre plate. The sensitivity was found to be 0.234 ng/ml after performing the student t-test.

4.3.3. Evaluation of different swabs

Different varieties of immunoswabs are available on the market. The best suited one was chosen out of five different swabs namely - nylon fibre swab, knitted polyester tipped swab, sterile cotton tipped swab, cotton tipped swab and calgiswab. The result is shown in Fig.4.7. The nylon fibre swab was found to be the best as it gave minimum colour in absence of any signal. So initially this nylon swab was selected for future work and subsequently the same nylon swab with comparatively less tip size was adapted because in the later version 50 µl volume of solution could completely immerse the swab tip whereas the corresponding volume for the former one was 150 µl. The selection of this last variety of swab helped to reduce the reagent volume in every step of the assay and this fact finally contributed towards the reduction of the material cost for the entire assay.

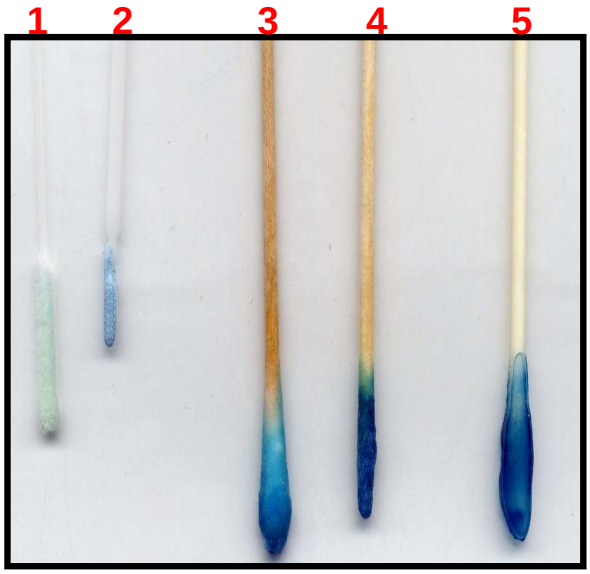


Fig.4.7. Testing of different commercially available swabs. 1. Nylon fiber swab from Micro Rheologics. **2.** Knitted polyester tipped swab from Puritan. **3.** Sterile cotton tipped swab from Pur-Wraps. **4.** Cotton tipped swab from Puritan. 5.Calgiswab from Spectrum.

4.3.4. Selection of blocking buffer for swab

After selecting the swab, different blocking conditions were tested to determine the best blocking condition for the swab assay. The swabs were blocked using different blocking buffers such as 5% BSA, 5% FBS, 5% Gelatin, 5% Skim milk, and a commercially available blocking buffer (blocking buffer supplied with commercially available ELISA kit). The swabs were incubated at room temperature for 45 min. All of the dilutions were made with PBS and two swabs were tested in each condition. After developing the colour with the substrate, it was found that, 5% BSA worked best as it gave a clear background. The result is shown in Fig 4.8.

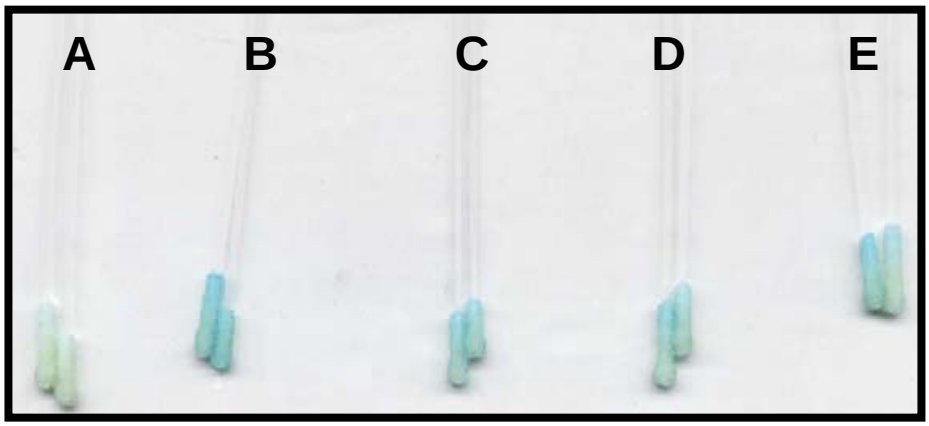


Fig 4.8. Assay done after blocking the swabs with different blocking buffers.
A- 5% Bovine Serum Albumin;**B**- 5% Fetal Bovine Serum; **C**- 5% Gelatin; **D**- 5% Skim Milk; **E**- commercial blocking buffer.

4.3.5. Comparison of the immunoswab assay in biotin labelled MAb and bsMAb format

In order to compare the sensitivity of the biotin labelled CS-35 and bsMAb as detection antibody, the immunoswab assay was performed with both following the protocol mentioned before in the method section. The assay was performed in PBS buffer media. The assay using the biotin labelled CS-35 gave higher background and the limit of detection of the antigen was found to be 5.0 ng/ml (Fig 4.9.A). While, the assay using bsMAb, the corresponding limit of antigen detection was found to be 0.005 ng/ml with a clear background (Fig 4.9.B).

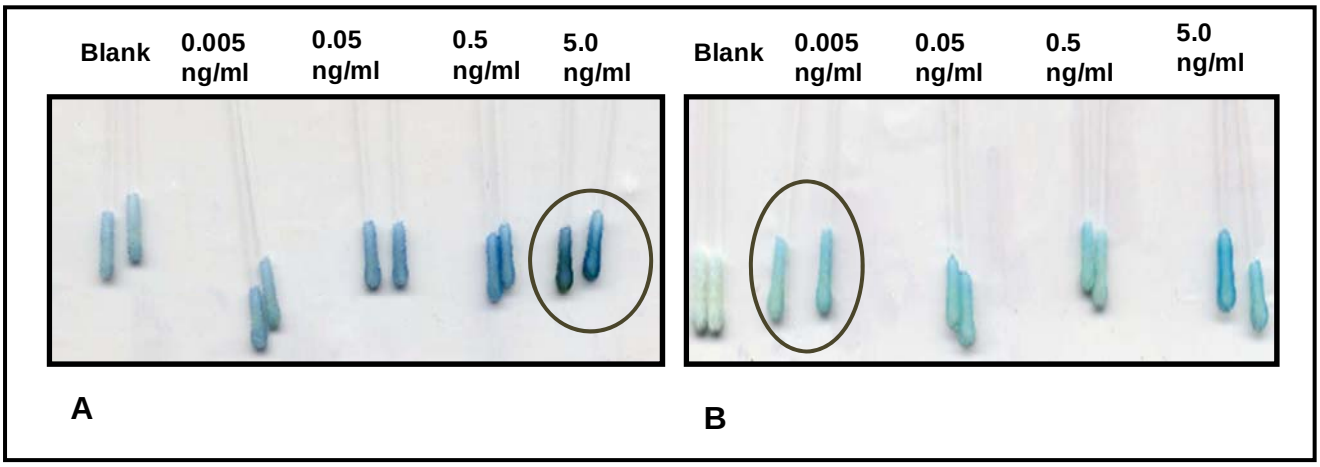


Fig 4.9. Immunoswab performed with biotinlabeled MAb (A) and bsMAb (B). The corresponding antigen concentration is mentioned at the top and the respective limit of antigen detection is encircled in the Figure.

The comparatively higher sensitivity (lower limit of detection) in the bsMAb format is partly because of the clear background which made it possible to distinguish the colour gradation visually at the lower level of antigen when compared with the blank. The higher background in the biotin labelled CS-35 can be explained by the fact that chemical conjugation occurring during biotinylation of CS-35 MAb is a random process wherein one molecule of the MAb may bind to more than one biotin molecule, which, in turn, will capture St-HRPO and give off enhanced signal leading to a higher background. In contrast, the combination ratio of bsMAb and HRPO is always 1:1 since bsMAb has only one paratope (antigen binding site) that can recognise HRPO, eliminating the possibility of enhanced signal resulting in a more clear background. Because of the higher sensitivity in the bsMAb format, it was chosen for the subsequent study.

4.3.6. Antigen detection limit in different matrices

Initially, the assay was performed with the antigen spiked in PBS buffer, but latter different body fluids were chosen to spike the antigen. Saline, rabbit serum and bovine urine were selected to spike the antigen. The limit of detection of BSA-Hex antigen spiked in saline was found to be 0.005 ng/ml (Fig. 4.10. saline). The same was found to be 0.5 ng/ml and 5 ng/ml in serum and urine respectively (Fig. 4.10. serum & urine). Two swabs were tested in each concentration.

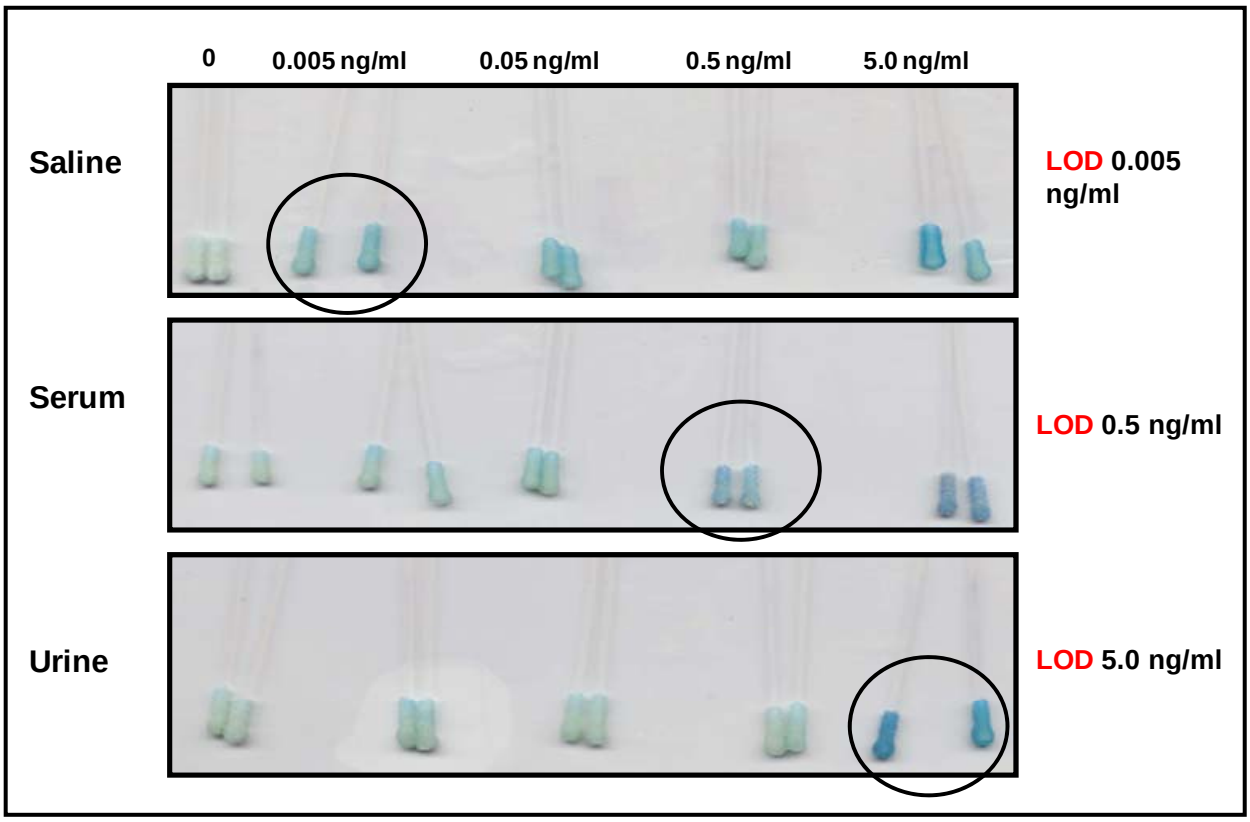


Fig 4.10. Immunoswab assay performed after spiking the BSA-Hex antigen in different fluids. The respective fluids are mentioned at the left side of the Figure and corresponding limit of detection (LOD) is mentioned at the right side, the LODs are also encircled in the Figure.

4.3.7. Assay sensitivity with the bacterial antigen

To check whether our designed assay can also detect the native antigen, the assay was evaluated using the bacterial LAM. The antigen was spiked in the serum and then the immunoswab assay was performed. The limit of detection was found to be 0.5 ng/ml. The result was shown in Fig 4.11. The evaluation of the assay with the bacterial antigen confirmed the feasibility of the assay to detect TB.

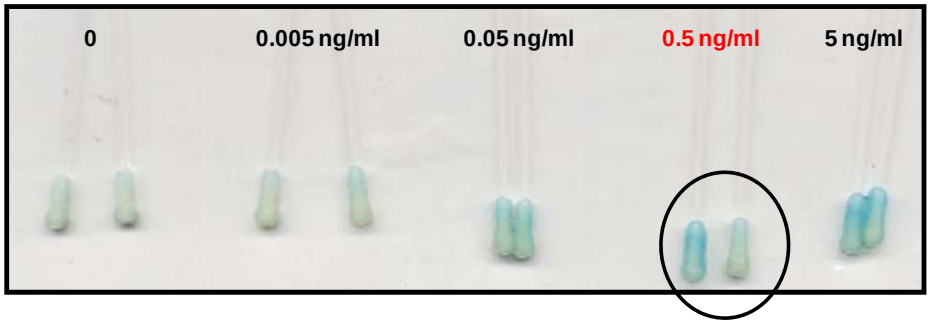


Fig.4.11. Immunoswabs performed with TB-LAM spiked in the rabbit serum. The LOD was found 0.5 ng/ml and the corresponding swab is encircled.

4.3.8. Assay specificity with different saccharides

The assay specificity was determined by spiking different saccharides in the PBS buffer. Since the target antigen, LAM, is a polysaccharide, two different saccharides were used as a non specific antigen. The first non specific antigen was sucrose and the second one was the human blood group-A trisaccharide conjugated to BSA. Since the specific antigen of this study, the hexasaccharide (a redundant epitope on LAM) is conjugated with BSA so the second non-specific antigen was also conjugated with BSA to provide structural homology.

The designed immunoswab assay clearly identified the specific BSA-Hex and gave a strong blue colour. Almost no colour development was found in the other two cases. The result is shown in Fig. 4.12. The result established the specificity of the assay towards the specific BSA-Hex antigen when compared with two other non specific but structurally related antigens (sucrose and BSA-conjugated to the human blood group A trisaccharide).

Fig. 4.12. The immunoswab done with different saccharides. 1. Blank (PBS); 2. BSA conju human blood group A trisaccharide; 3. Sucrose; 4. BSA-Hex (specific antigen)

4.3.9. Cross reactivity with different bacterial and synthetic antigens

The blue coloured dot was developed in five places - BSA-Hex, TT-Hex, TB-LAM, Lep-LAM and Smeg-LAM, but not in BSA in both the assay using biotin labeled CS-35 and bsMAb. The results were similar in both the cases so only the dot blot with the biotin labeled CS-35 was shown in Fig 4.13. This test showed that both our MAb and bsMAb were cross reacting with the other mycobacterial LAM. This is expected as the structure of LAM is almost comparable among the mycobacterial species. Due to limited availability of the bacterial LAM, it was not possible to do the test with the immunoswabs. But based on this result, we can conclude that the designed assay will show cross reactivity with other mycobacteria as we used same CS-35 and bsMAb in our assay.

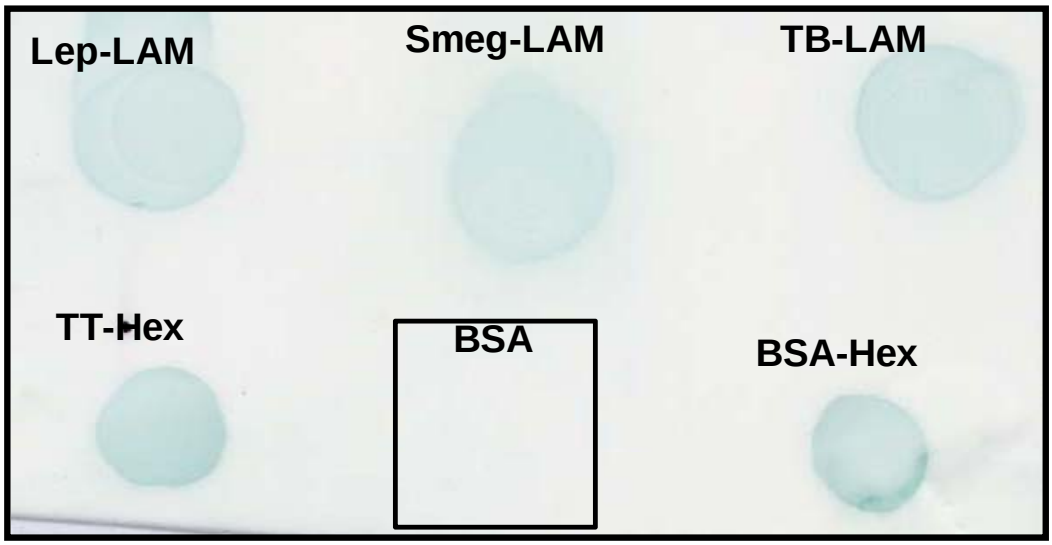


Fig. 4.13. The dot blot assay with different antigens. Three different bacterial LAMs (Lep-LAM, Smeg-LAM and TB-LAM) and two carrier conjugated synthetic antigen (TT-Hex and BSA-Hex) along with BSA were used to check the cross reactivity of the antibody of interest, CS-35. No dot was found with BSA (highlighted part).

The assay designed in this chapter was capable of detecting the TB antigen from different fluids. During the TB infection, the LAM antigen was found to be present in sputum (Arias-Bouda et.al 2000), serum (Sada et.al 1992) and urine (Boehme et.al 2005). To mimic these human body fluids; saline, rabbit serum and bovine urine were used. In terms of limit of detection, the designed immunoswab assay showed comparable sensitivity with the earlier asaays developed in our lab (Kammila *et al*. 2008 and Tang *et al.* 2004).

Chapter V. Evaluation of the assay with clinical samples

5.1. Introduction

The final objective of this study was to develop an immunoassay that could detect TB at the point-of-care level. Accordingly, the required antibodies were developed, purified and characterized. Subsequently the proposed assay was developed by using these antibodies. The designed assay could detect the TB antigen, LAM, from serum and urine when the antigen was spiked in them. The sensitivity and specificity of our designed assay was fairly good. The end point of the assay could be read out visually without the requirement of using any sophisticated instrument and the result can be obtained within 2 hours of sample collection. Another advantage of our assay was that it detects the antigen and as it is well known that detection of antigen in any body fluid confirms the recent exposure to the disease (Pai et al., 2006).

To check the reliability of the assay in detecting the actual clinical samples, the assay was evaluated with different serum samples. There are different groups of TB infected patients - latently infected, actively infected, HIV co-infected, disseminated TB and those who have already undergone some phase of antibiotic treatment. Here, we worked with the stored clinical samples collected from non-HIV cavitary TB infected patients.

The assay was first evaluated on the direct samples without any prior treatment. Sometimes because of the formation of antigen-antibody complex (especially in serum),

practically very low antigen is available for detection so later we tried to apply different simple processing techniques to enrich the free antigen content.

5.2. Materials & Methods:

5.2.1. Materials

The purified MAb CS-35, bsMAb and the biotin labelled CS-35 were processed in our lab as per the previously described procedure. Streptavidin tagged HRPO (St-HRPO) and St-alkaline phosphatase were purchased from BD Biosciences (California, USA). The rabbit anti-human IgG-HRPO and p-Nitrophenyl Phosphate Liquid Substrate System (substrate for alkaline phosphatase) were purchased from Sigma chemicals (St.Louis, USA). Tetramethyl benzidine (TMB) substrate was purchased from BioFx Laboratory (North Carolina, USA. Streptavidin tagged alkaline phosphatise was procured from BD Bioscience (New Jersey, USA). Nylon fibre swab was bought from Micro Rheologics (Brescia, Italy). Non-sterile flat bottom NUNC maxisorp 96-well ELISA plates were purchased from VWR (Ontario, Canada). All plastic supplies and dialysis bags were purchased from Fisher Scientific (Houston, USA). The centrifuge model used was MSE Mistral 2000. For protein purification, we used Biologic Duoflow system (Bio-Rad, USA) and the ELISA absorbance was taken using Versa max microplate reader (Molecular devices, USA).

The clinical samples were procured from Dr. John Spencer, Departments of Microbiology, Immunology and Pathology, Colorado State University, USA. The positive samples were collected at different time points during 1995-1998, from non HIV-TB patients (The Tuberculosis Trial Consortium, 2002). The negative samples were collected from healthy

volunteers from non endemic area around 2002 (sample number suffixed with NS). The samples were assigned positive and negative in terms of anti-LAM titre (Spencer et al., 2011).

The antigen used was a chemically-synthesized hexaarabinofuranoside epitope of the LAM antigen conjugated to BSA (BSA-Hex). Native LAM from *M. tuberculosis* H37Rv strain was obtained from the Biodefense and Emerging Infections Research Resources Repository Manassas, Virginia, USA.

5.2.2. Dilution of the sample

The serum samples were received in dry ice shipment and immediately stored at -80°C upon receipt. They were thawed before using and diluted in the PBS buffer (containing 1% dialysed BSA) as required. The dilution ratio was mentioned and when required in the subsequent sections.

5.2.3. Antigen Detection

5.2.3.1. The assay with the immunoswab

This testing was performed in almost same way as the described before (section 4.2.6). Briefly, the swabs were first coated with CS-35 MAb (50 µL of 25 µg/ml in PBS) at RT for 30 minutes, dried for 5 minutes and fixed with 50 µL of 95% ethanol for 1 min. The swabs were then blocked with 5% DBSA in PBS at RT for 45 minutes and were washed five times using PBST with 1 minute incubation in each washing step. The washed swabs were incubated for 30

minutes with different clinical samples for 30-45 min. Subsequently, the swabs were washed as before and incubated for 30 minutes with the bsMAb (50 µL of 10 µg/ml in PBS containing 1% DBSA). Next the swabs were washed five times as mentioned above and finally 100 µL of TMB was added for colour development. The diluting media was used as the blank of the experiment. Following colour development, the swabs were scanned using an Epson scanner.

This assay was performed with 1:10 dilution of the clinical samples. Each sample was tested in duplicates.

5.2.3.2. The assay in the microtitre plate using HRPO

The assay was repeated in the microtitre plate. In this case, we used biotin tagged CS-35 for detection instead of bsMAb. The assay protocol was almost similar as described in the earlier chapter (section 4.2.2.2). Briefly, the microtitre plate was coated with 8 µg/ml of CS-35 and incubated overnight at 4°C. After washing, the blocking step was performed with 250 µl of 3% DBSA in PBS at 37°C for 3 hours. The plate was washed 3 times with PBST and then 100 µl of diluted clinical was added and incubated for 2 hours, and washed. Subsequently 4 µg/ml of biotin labelled antibody (DAb) was added and incubated for 1 hour and the plate was washed. To this St-HRPO (1: 8000 dilution) was added and incubated for 30 minutes. The plate was washed 3-5 times with PBST. Finally TMB substrate was added and the developed color was read at 650 nm using the micro titre plate reader.

This assay was performed with different dilutions of the clinical samples like 1:5. 1:10, 1:100 and 1:200. Each sample on each dilution was tested in triplicates.

5.2.3.3. The assay in the microtitre plate using alkaline phosphatase (AP)

The assays reported above were done with the HRPO enzyme, next, we tried to test the samples with the alkaline phosphatase (AP). The assay protocol was exactly same as described above. The only exception was that instead of adding St-HRPO, we used St-AP (1:8000) and finally the corresponding substrate for AP was added to develop the colour which was read at 405nm.

This assay was performed with 1:10 dilution of the clinical samples. Each sample was tested in triplicates.

5.2.3.4. Simulation of clinical samples with rabbit serum

In order to check whether the presence of specific antibody reduces the antigen detection ability we simulated the clinical samples with the rabbit serum (RS). First, CS-35 antibody was added to the rabbit serum at two different concentrations – 10 μg/ml and 1 μg/ml and then BSA-Hex antigen was added at a concentration of 5 ng/ml. The samples were kept on rocker at 4°C for overnight. Then the assay was performed to detect the antigen. The control of the study was made by spiking 5 ng/ml BSA-Hex in rabbit serum without any CS-35 antibody.

5.2.3.5. Processing the samples in different conditions

The above simulated samples were treated with different processing conditions. Here, the simulated samples were made as above. But in this case, we spiked the CS-35 at three different

concentrations – 20 µg/ml, 2 µg/ml and 0.2 µg/ml. TB-LAM was used as antigen at a concentration of 0.5 ng/ml in all the cases. The different conditions were mentioned in Table 5.1. After processing the samples were tested for the antigen level. The entire reduction and subsequent antigen detection assay was performed with three different antibody concentrations – 20 µg/ml, 2 µg/ml and 0.2 µg/ml. Each condition in each antibody concentration was tested in duplicate.

Table 5.1. Different processing conditions for simulated samples. The abbreviations used, RT=Room temp, SDS=Sodium dodecyl sulphate, β-ME= β-mercaptoethanol. For all the conditions the serum used was 130 µl.

Conditions	Chemicals used	Temperature & time
1. No treatment	Water = 50 µl	RT for 10 min
2. Glycine + Tris	Glycine (0.1M, pH 2.3) = 40µl Tris(1M, pH 9.0) = 10 µl	RT for 10 min, First glycine was added and incubated for 5 min then neutralised back with Tris
3. Urea + heat	Urea (8M) = 50 µl	93°C for 5 min and immediately cooled to 4°C.
4. SDS + heat	SDS (10%) = 20 µl Water = 30 µl	93°C for 5 min and immediately cooled to 4°C.
5. SDS + β-ME+ heat	SDS (10%) = 20 µl Water = 28 µl β-ME = 2 µl	93°C for 5 min and immediately cooled to 4°C.

5.2.3.6. Chemical processing of the clinical samples

The clinical samples were diluted 1:8 and then each sample was treated as per condition 3 mentioned in Table 5.1. Then the assay was performed as explained before. Each sample was tested in duplicate.

5.2.4. Antibody detection

The assay protocol was same as described in the earlier chapter (2.2.3). Briefly, the plate was coated with 100 µl of the BSA-Hex antigen (8 µg/ml) in coating buffer at 4°C overnight. The microtitre plate was washed three times with phosphate buffer saline containing 0.05% tween-20 (PBST; pH 7.4) and the unoccupied binding sites were blocked using 200 µl of 3% BSA for 3 hours at 37°C. After washing three times with PBST, the 1:100 diluted clinical samples (50 µl) were added and incubated for 2 hours at RT. Subsequent to washing three times with PBST, rabbit anti-human IgG-HRPO (1:10,000 dilution) was added and the plate was incubated for 30 minutes at RT. Finally, the plate was washed at least five times with PBST then TMB substrate was added and the developed blue color was read at 650 nm using the microplate reader.

5.3. Results and Discussions

5.3.1. The actual identity of the clinical samples (assay performed by the Colorado State University)

The **actual identity** of the samples is described in **Table 5.2**. This test was performed at Colorado State University and based on their findings, the total 21 samples were divided into three categories – negative (7 samples), weak positive (4 samples) and strong positive (10 samples). **However**, here the samples were analysed in terms of the anti-LAM antibody but our goal was to detect the LAM antigen. Though it is difficult to correlate the concentration of antibody with that of antigen, but we tried to evaluate the samples assuming the direct correlation between them. So, negative samples mean no antigen, weak positives mean less antigen and strong positives mean the highest antigen.

Table 5.2. The actual identity of the clinical samples (results obtained from the Colorado State University). The samples were analysed on the basis of anti-LAM antibody present on them.

	Negative	**Weak Positive**	**Strong Positive**
Sample No.	NS1, NS2, NS3, NS4, NS5, NS9 and NS14 **Total 7 samples**	206374, 207761, 209250 and 213603 **Total 4 samples**	206106, 209243, 206363, 209246, 223470, 222090, 225845, 212190, 212194 and 216894 **Total 10 samples**

5.3.2. Antigen detection

5.3.2.1. With the immunoswab

The clinical samples (1:10 diluted) were tested with immunoswabs using the previously optimized conditions. The result is shown in Figure 5.1. After comparing the colour intensity with that of the blank, it was difficult to confirm the identity of the samples. So it was planned to repeat the assay in the microtitre plate. Moreover, almost no correlation could be traced between the immunoswab result and Table.5.2.

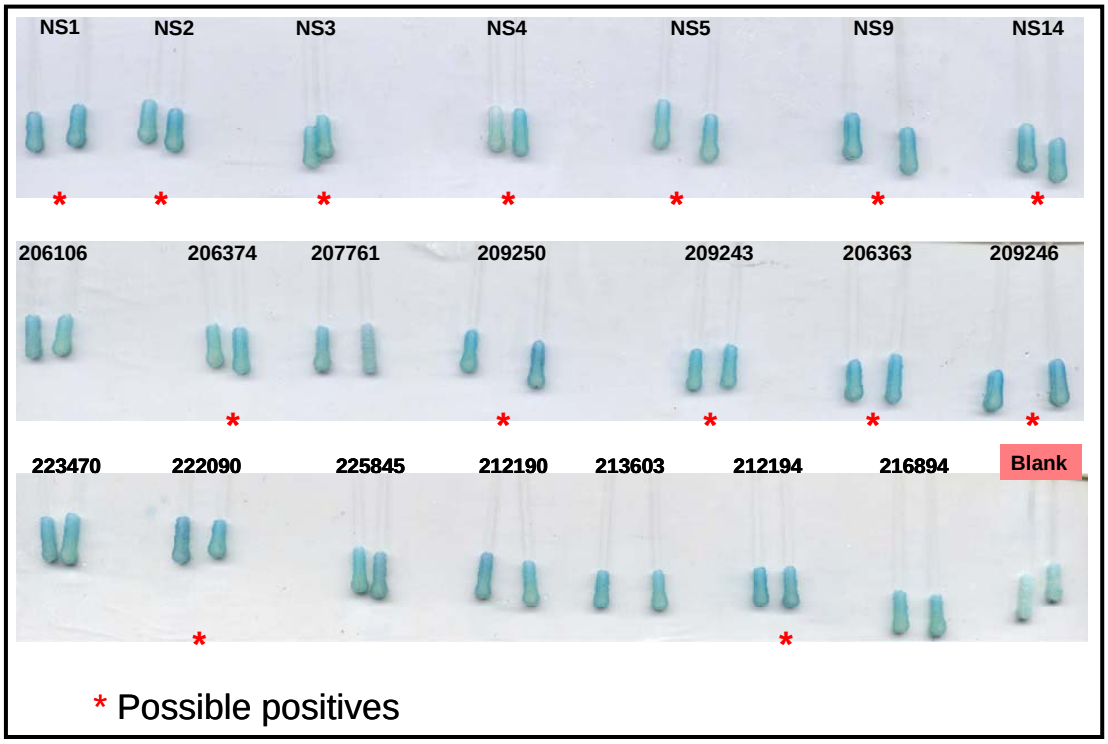


Figure. 5.1. Immunoswab assay performed with 1:10 diluted clinical samples. The possible positives are marked "*".

5.3.2.2. In the microtitre plate using HRPO

The clinical samples were analysed after diluting 1:5, 1:10, 1:100 and 1:200. The results of first two dilutions are shown in Figure 5.2.A and that of 1:100 was in Figure 5.2.B. The result for 1:200 dilution was similar to that of 1:100 so it is not shown. After comparing Figure 5.2.A with Table 5.2, almost no correlation is found since the negative samples were giving higher signal compare to the positives. In the higher dilutions, (Figure 5.2.B), almost all the samples were giving same signal.

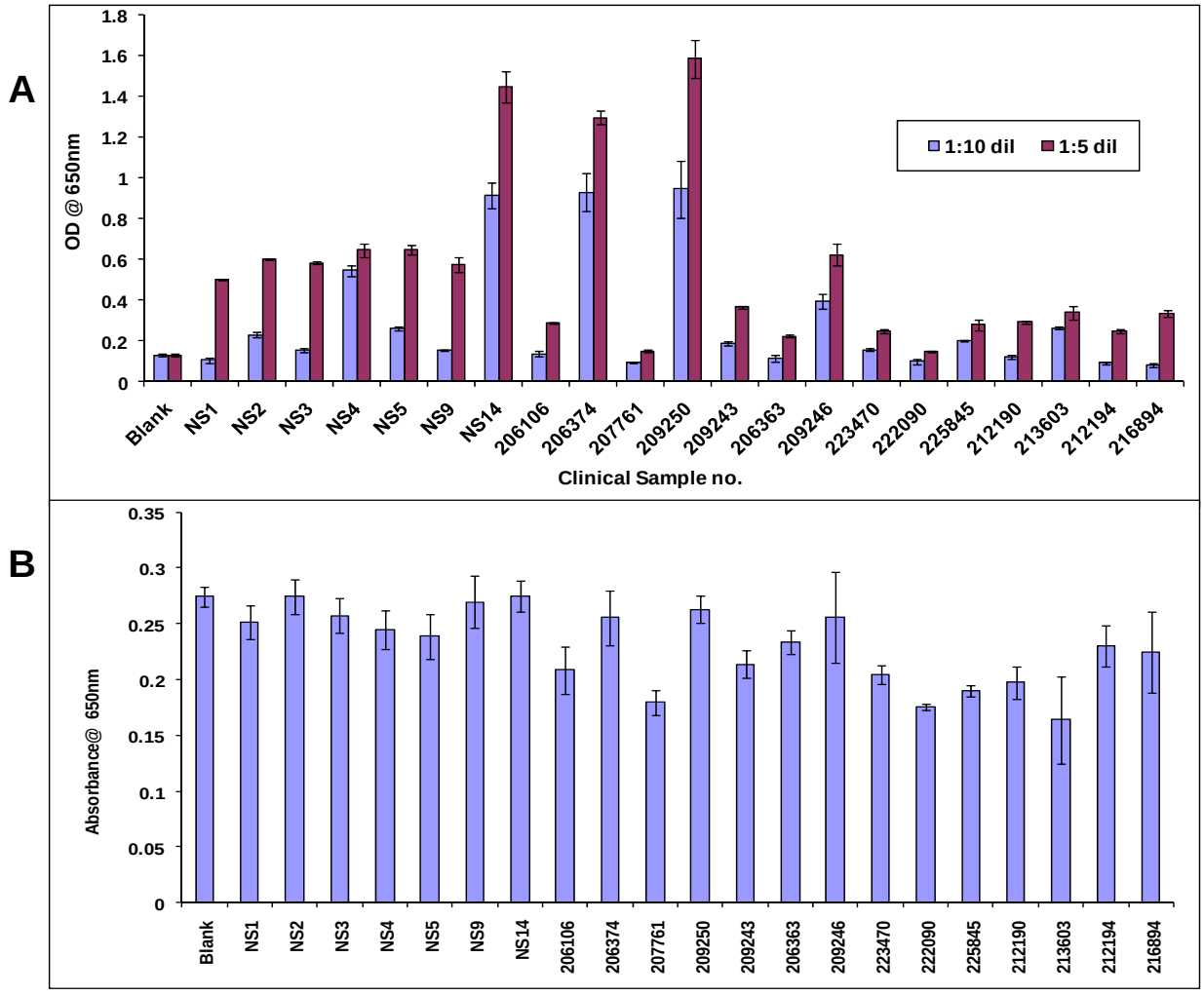


Figure 5.2. The antigen detection result in the diluted clinical samples. A= Samples diluted 1:5 and 1:10. B= Samples diluted 1:100.

5.3.2.3. In the microtitre plate using AP enzyme system

None of the above mentioned attempts were able to give the expected result that corresponds with the Table 5.2. Up until now, the HRPO enzyme was used. So in order to check that HRPO was not interfering in the result, the assay was done using a second enzyme and corresponding substrate. The second enzyme chosen was the alkaline phosphatase (AP). The samples were tested only in 1:10 dilution and the result was shown in Figure. 5.3. along with the corresponding HRPO data at the same dilution. From the Figure 5.3 it was clear that except in 5 cases (tick marked in the Figure) the rests showed the same trend in both the enzyme systems.

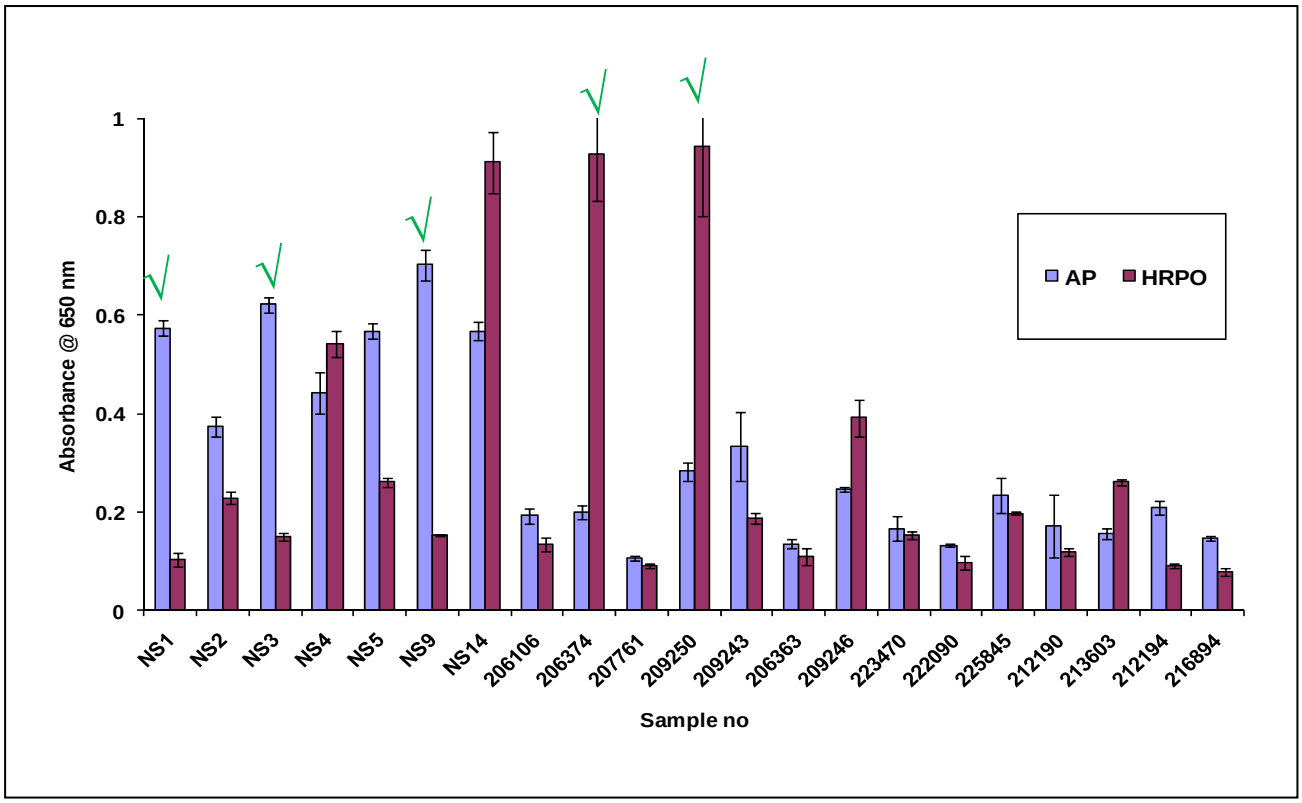


Figure 5.3. The samples tested in two different enzyme systems. The serum dilution was 1:10 and the enzymes used were horseradish peroxidase (HRPO) and alkaline phosphatase (AP). The tick marked ones showed the discrepancy in two systems.

5.3.2.4. Simulation of clinical samples

In order to check the effect of the presence of antibody in the detection of antigen, the condition of clinical samples was simulated in the rabbit serum and the result was shown in Figure 5.4. The results clearly showed a suppression of antigen signal in presence of antibody. Since excess of antibody (10 µg/ml and 1 µg/ml) was used compare to the antigen (5 ng/ml) so the degree of signal suppression was almost similar under the two different antibody concentrations. As shown earlier that the sensitivity in serum was found to be 0.5 ng/ml both in terms of BSA-Hex and TB-LAM (section 4.3.6 and 4.3.7). So here the antigen was used at 5 ng/ml to ensure that the antigen content was well within the limit of detection of the designed assay.

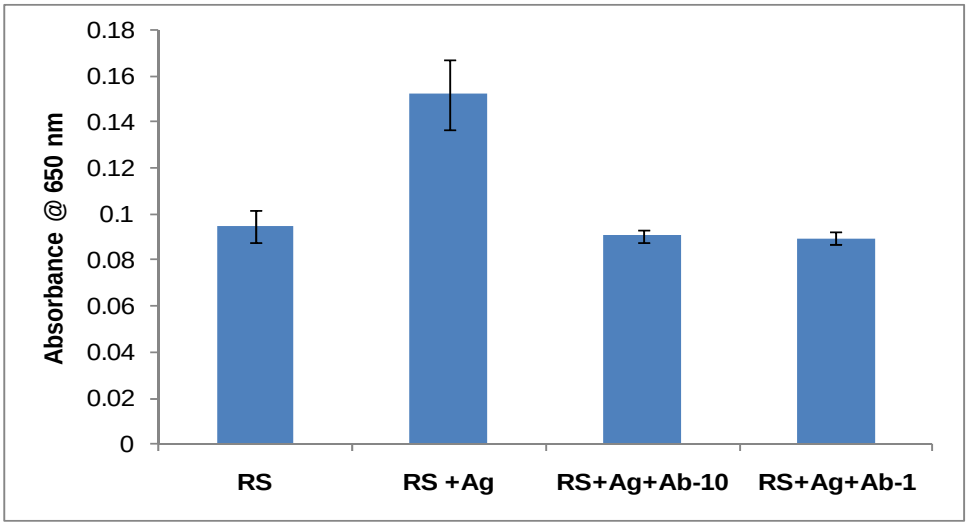


Figure 5. 4. The effect of the presence of antibody (Ab) on the antigen (Ag) detection. The clinical sample condition was simulated in the rabbit serum (RS). Two different Ab concentrations were used, Ab-10 (10µg/ml CS-35) and Ab-1 (1µg/ml CS-35). The Ag used was BSA-Hex (5ng/ml).

5.3.2.5. Processing the samples under different conditions

The above finding necessitated the processing of the samples in order to dissociate the antigen-antibody immune complex (IC). Different techniques were tested, as mentioned in Table 5.1, to check which one of them could dissociate the IC. The idea behind choosing different conditions was either acidification would facilitate the dissociation of antibody-antigen complex (as tested in glycine+tris condition) or reduction or denaturation of the antibody would free the antigen from the IC (as tested by the other conditions). In contrast to the previous section, here TB-LAM was used as antigen because we thought that different treatment conditions might affect the stability of BSA-Hex. The result was shown in Figure 5.5. The result showed that urea + heat worked best to dissociate the IC among all the conditions.

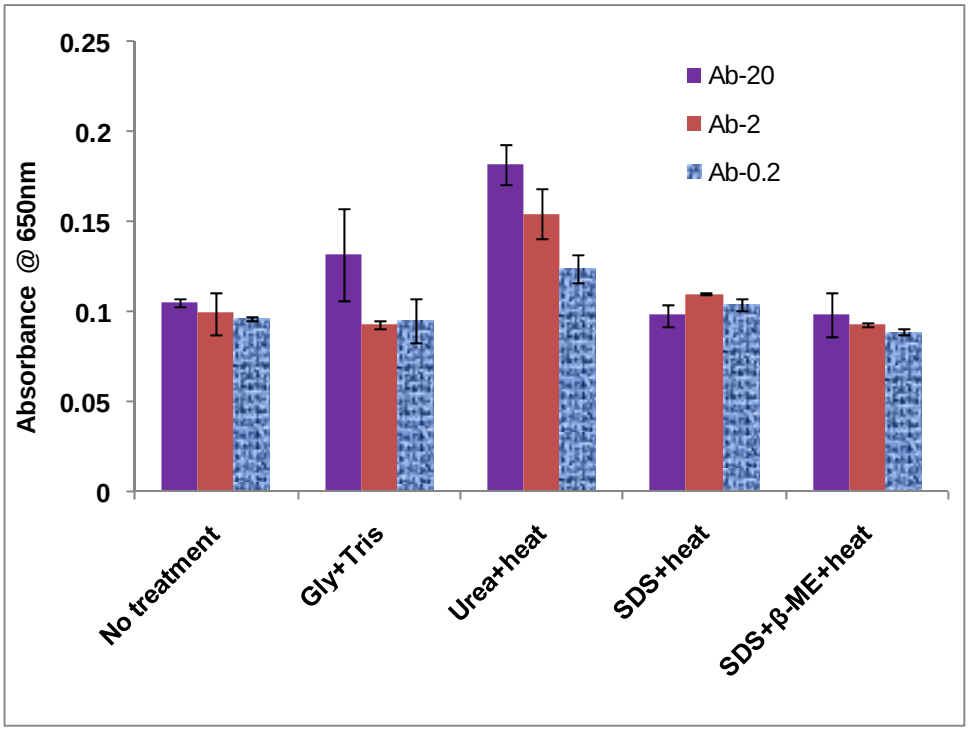


Figure 5.5. The recovery of antigen detection signal after different chemical treatments on the simulated clinical samples. Different conditions are mentioned in x-axis. Three different antibody (Ab) concentrations are used as mentioned in the top right side.

From the above Figure, it was clear that urea and heat treatment was able to dissociate the antibody-antigen (Ab-Ag) immune complex (IC). Although SDS and β-mercaptoethanol both are potent protein denaturing and reducing agents like urea but we got best result only with urea. This could be explained by the fact that the excess reagent was not removed before doing the immunoassay and the presence of SDS in the processed sample was inhibiting the Ab-Ag attachment in our subsequent assay. But excess urea was not affecting the result because of the fact that urea denatures protein only at elevated temperature (Bennion and Daggett, 2003) and we conducted our immunoassay at RT. As far as the glycine+tris condition was concerned, there is at least one report in literature that showed the glycine+tris treatment can dissociate Ab-Ag complex but 1 hr of incubation is required (Vasudevachari et al., 1993). But we processed the samples only for 10 min so probably the time was not enough to dissociate the complex. Based on our above finding, urea and heat treatment was selected to process the clinical samples.

5.3.2.5. Chemical processing of the samples

Since the urea+heat treatment was able to efficiently dissociate the Ab-Ag complex, So the clinical samples were treated with urea+heat condition and the tested for antigen level and the result was shown in Figure 5.6. After comparing this finding with Table 5.2, good correlation was found. Based on the mean absorbance value of the negative samples the cut of point was chosen based on the mean + 2SD (standard deviation) (the blue line in the figure). The specificity of the assay was found to be 100% as all the negative samples lie below the cut of line. And, out of 14 positives (strong + weak) 9 was found to lie above the cut of line, leading to

a sensitivity of 64% in this case except that two of the strong positive samples (206106 and 206363) were giving less signals and one of the weak positive sample (213603) was giving strong signal.

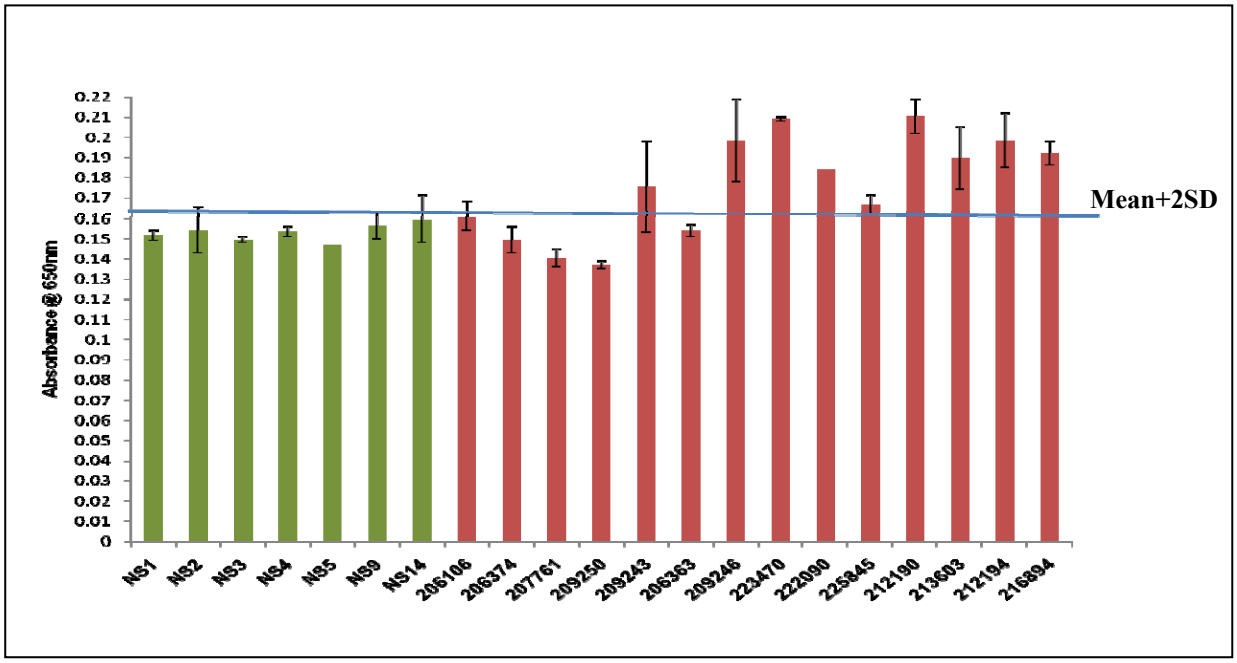


Figure. 5.6. The level of antigen in the clinical samples after urea and heat treatment. The samples were diluted 1:8. The blue line indicate the cut-of-line. All the negative samples (green bar) lied below the blue line whereas 9 out of 14 positive samples (red bar) lied above the line.

5.3.3. Antibody detection

Finally we tried to test the samples in terms of anti-LAM antibody content. The antigen used to detect the antibody was BSA-Hex antigen so the control of the study was BSA. Due to availability of limited quantity of samples, the assay was done in single well per sample. The

absorbance data were shown in Table 5.3 and the graphical representation was shown in Figure 5.7.

Table 5.3. The absorbance value of the clinical samples on the BSA-Hex and BSA coated plate. We found 10 positive (shown in bold) and rests negative.

Sample No.	BSA-Hex (A)	BSA (B)	Differential absorbance (A-B)
Blank	0.068	0.029	0.039
NS1	0.433	0.332	0.101
NS2	0.379	0.337	0.042
NS3	0.202	0.166	0.036
NS4	0.508	0.514	-0.006
NS5	0.379	0.442	-0.063
NS9	0.278	0.343	-0.065
NS14	0.238	0.173	0.065
206106	1.945	0.317	**1.628**
206374	0.511	0.502	0.009
207761	0.558	0.274	**0.284**
209250	0.361	0.318	0.043
209243	1.71	0.429	**1.281**
206363	0.74	0.76	-0.02
209246	0.653	0.379	**0.274**
223470	2.779	0.172	**2.607**
222090	3.017	0.233	**2.784**
225845	1.53	0.26	**1.27**
212190	2.695	0.583	**2.112**
213603	0.417	0.381	0.036
212194	2.794	0.327	**2.467**
216894	2.956	0.832	**2.124**

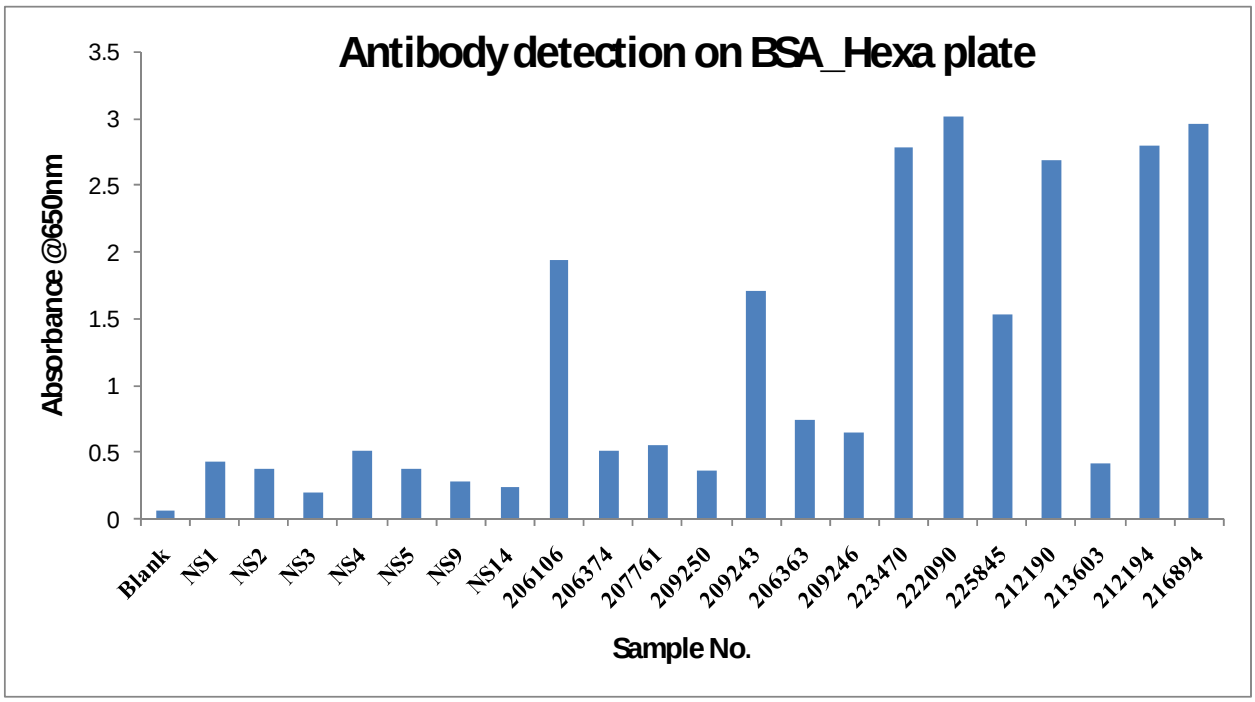


Figure. 5.7. The result of antibody detection in the 1:100 diluted serum

After comparing our findings in terms of antibody level (Table 5.3) with that of the actual findings (Table 5.2), the followings were found:

- **For –ve samples**, specificity was **100%** [NS1-5, 9 and 14 were showing –ve as per both Table].
- **For +ve samples**, specificity was **71%** [10 out of 14].

  Please note, the 4 samples were v.weak +ve (as per Colorado State University, Table 5.2) so if we omit those then specificity is **90%** [9 out of 10].
- **False signal**, 4 (including all 21 samples)

  1 (Sample No. 206106) (excluding the 4 weak +ves).

From the above findings, it was clear that the strong positive samples contained huge amount of antibody and that might be the reason for getting so low antigen signal in our earlier attempts. However, the treatment of the samples with urea and heat was able to dissociate the antibody-antigen complex by reducing or denaturing the serum antibody. Since the target antigen LAM is a carbohydrate so this process is not affecting it and therefore we were able to detect the antigen after the treatment.

Chapter VI. Conclusion and future plan

6.1. Conclusion

6.1.1. Summary

TB is a highly infectious disease in its active state because the disease can be acquired by inhaling as low as ten bacteria (Lipman *et al*., 2005). If left untreated, an individual with active TB disease can potentially infect an average of 10–15 people per year (WHO TB factsheets). Early and rapid detection of TB is therefore extremely important to not only prevent the spread of the disease in the community but also ensure appropriate treatment for the affected individual.

The bsMAb developed in this study, showed dual binding specificity towards the TB antigen LAM and the enzyme HRPO. The immunoswab assay was evaluated in both the bsMAb and biotin labelled MAb format. As per the discussed result, the bsMAb format was found to be more sensitive than the other (section 4.3.5). The assay was also evaluated with the clinical samples but we could not detect the LAM from those samples prior to any processing, mostly because of the presence of humongous amount anti-LAM antibody in them. However, the antigen was detected after reducing the antigen-antibody complex with urea. Out of different conditions tried (listed in Table 5.1), the urea and heat treatment was able to efficiently dissociate the antibody-antigen (Ab-Ag) immune complex (IC). Although SDS and β-mercaptoethanol both are potent protein denaturing and reducing agents like urea but we got best result only with urea. This could be explained by the fact that we did not remove the excess reagent before doing our immunoassay, just to avoid extra processing step and time. The

presence of SDS in the processed sample was inhibiting the Ab-Ag attachment in our subsequent assay. But excess urea was not affecting the result likely because of the fact that urea denatures protein above 60°C (Bennion and Daggett 2003) and we conducted our immunoassay at room temperature. As far as the glycine+tris condition was concerned, there is at least one report in literature that showed the glycine+tris treatment can dissociate Ab-Ag complex but 1 hr of incubation is required (Vasudevachari et al., 1993). But we processed the samples for 10 min only, so probably the time was not enough to dissociate the complex. We selected the urea + heat condition to pre-treat the samples, and we were able to achieve good assessment of the clinical samples after this treatment. The processing technique, employed here, was very simple (just heating and cooling) and fast too (10 min treatment time).

In this study the LAM antigen of *M. tuberculosis* was the target for the development of an immunoswab assay to detect TB. Mtb secretes LAM to manipulate the host immune system (Józefowski *et al.,* 2008), and therefore detection of LAM can be efficiently done to detect the disease (Patel et al., 2010). LAM is abundant in the *M. tuberculosis* cell wall (Abebe *et al.,* 2007), and is primarily composed of mannose and arabinose sugars that are repetitive throughout the structure (Hunter *et al*., 1984; Chatterjee and Khoo 1998; Strohmeier and Fenton 1999; and Kaur *et al.*, 2002). LAM has been shown to be present in not only sputum but also in blood, urine and cerebrospinal fluid of *M. tuberculosis* infected patients (Sada *et al.,* 1992; Arias-Bouda *et al*., 2000; Boehme *et al*., 2005 and Patel et al., 2010). The occurrence of LAM in body fluids other than sputum especially facilitates the detection of extra-pulmonary TB. Also, compared to sputum collection, it is relatively easy to collect blood or urine samples, particularly from the elderly and paediatric populations who are the main victims of TB due to their weakened immune system. The immunoswab assay reported here is capable of detecting LAM spiked in

both serum and urine, indicating that the assay might be useful in detecting pulmonary, extra-pulmonary and disseminated TB. Initially the assay was done by using the synthetic epitope of LAM, named BSA-Hex, but latter the assay was evaluated with the bacterial antigens and the limit of detection was 0.5ng/ml in serum for both synthetic and bacterial antigens.

In one study reported by Arias-Bouda et al., they found a correlation between the quantity of LAM antigen secreted with the corresponding number of bacterial load (Arias-Bouda et al., 2000) and their finding is reproduced in Figure 6.1. According to them, 1 ng/ml LAM was equivalent to 10^4 bacteria/ml which is the sensitivity range for sputum smear microscopy (SSM). In our study, the limit of detection for bacterial LAM was found to be 0.5 ng/ml in serum so this assay is expected to be more sensitive than SSM.

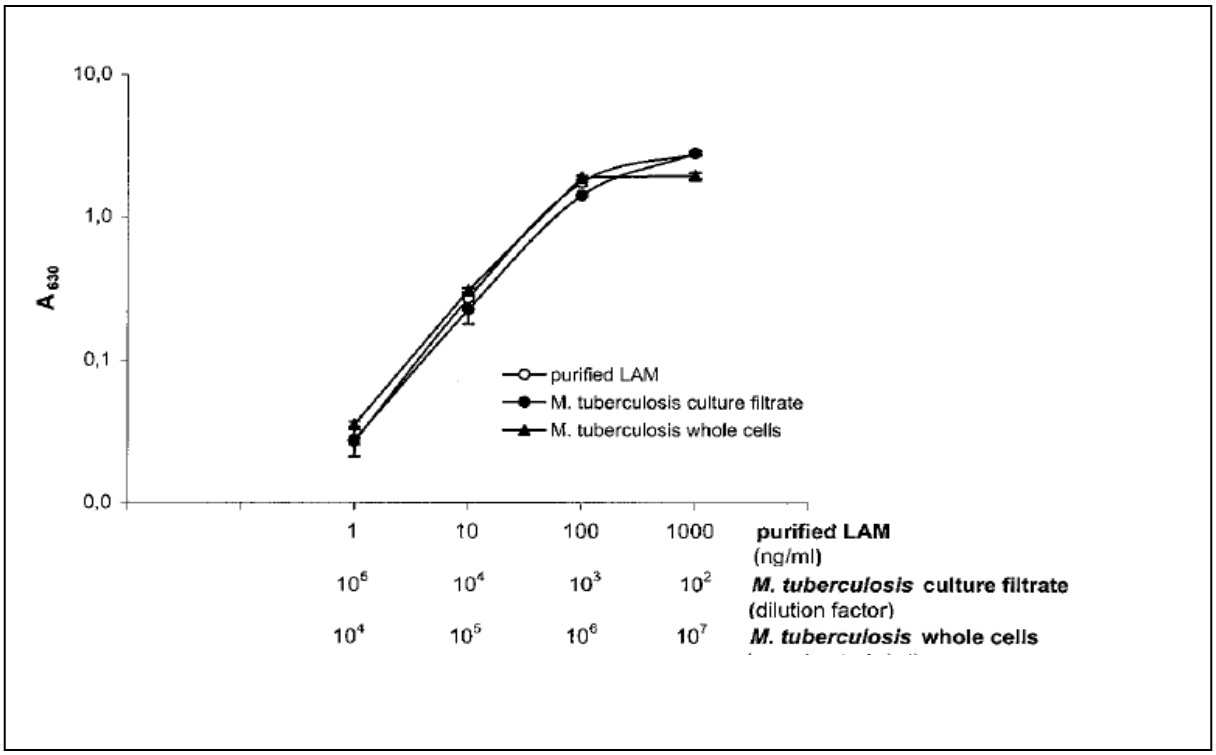


Figure.6.1. The correlation between the amount of LAM and the bacterial load. The figure was adapted from Arias-Bouda et al.,2000, *J of Clin Microbl*.38: 2278-2283.

The assay was also evaluated with structurally related carbohydrate moieties like, sucrose and nonspecific trisaccharide and strong specificity was found towards the specific BSA-Hex antigen. Though the antibodies showed cross reactivity with related LAM molecules from non-tuberculin mycobacteria but that should not restrict the assay application. Since 70% of the AIDS patients are infected with at least one of the opportunistic mycobacteria (Inderlied et al., 1993). Moreover, the specificity was found to be 100% when tested with the clinical samples.

The assay result could be obtained within 2 hours of the sample collection. The stability study was performed by storing the swabs at different conditions like 37°C, 4°C and -20°C after the coating and blocking stage. The result showed no change in sensitivity even at 37°C storage for 2 weeks, the sensitivity was reduced after that (data not shown).

A rough estimation was made regarding the reagent cost per swab. The cost was found to be below 1 CAD, which includes the cost of coating, detection antibody and the buffer chemicals (cost analysis not shown). The end point of the assay can be read out visually without the need of any instrument. Moreover, the assay protocol described in this study can be performed by the personnel with minimal technical expertise. So in terms of time, cost and technical expertise our designed assay can be regarded as user friendly. The relative ease and robustness of this immunoswab assay makes it an useful tool to detect infectious diseases at different points of human entry such as airports, seaports, and bus and train stations (Kammila et al., 2008). Screening at the points of entry can immensely help to quarantine affected individuals coming from the high TB burden countries and thereby minimize the spread of the disease.

In conclusion, the designed assay has all the potentials to serve as an ideal point of care (POC) detection for TB as the assay is affordable, sensitive, specific, user-friendly, rapid and

robust, equipment-free and therefore can be delivered to those in need. All of these parameters, acronymed as ASSURED, were identified as the ideal characteristics for a diagnosis test (Mabey, 2004).

6.1.2. Limitation

The assay might show cross reactivity with non tuberculin mycobacteria (NTM). As shown earlier (in section 4.3.9), both the bsMAb and CS-35 showed binding towards the different mycobacterial LAM (*Mycobacterium smegmatis, Mycobacterium leprae* and *Mycobacterium tuberculosis*). It was found to be showing reactivity with all of them and that is expected due to the structural homology of LAM among them. This might be the limitation of the assay but at the same time studies had shown that 70% of the AIDS (acquired immunodeficiency syndrome) patients were infected with at least one of the opportunistic mycobacteria pathogens (Inderlied *et al.,* 1993). So the designed assay might come useful to predict those cases. Moreover, the relative abundance of the antigens, secreted from the non-pathogenic strains, was expected to be less in different body fluids for immune competent host.

6.2. Future plan

Three future directions can be suggested from this study:

- The assay can be evaluated to test the clinical samples collected from different groups of TB affected population like – latently infected, actively infected, HIV co-infected, disseminated TB etc. This will help to determine the suitable groups of TB affected

population that can benefit from this assay. The assay has to be evaluated with both serum and urine samples. As often times the presence of antibody in serum may confound the antigen detection result so in that respect urine may be a good alternative. Moreover, urine sample collection is non invasive.

- The rapid emergence of multi drug resistant (MDR) and extremely drug resistant (XDR) TB bacteria necessitates a rapid and point of care detection for them. So similar kind of assay can be designed to detect the MDR and XDR forms of TB by targeting the specific antigens.

- In order to rule out the cross reactivity issue, similar kind of assay can be designed using the noble TB specific antigens like CFP-10 (culture filtrate protein 10) and ESAT-6 (early secretory antigenic target 6)

References:

Abebe F, Holm-Hansen C, Wiker H G and Bjune G. (2007). Progress in serodiagnosis of *Mycobacterium tuberculosis* infection. *Scand. J. Immunol*. 66: 176-191.

Abe C, Hirano K, Wada M, Kazumi Y, Takahashi M, Fukasawa Y, Yoshimura T, Miyagi C and Goto S. (1993). Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified *Mycobacterium Tuberculosis* Direct Test. *J. Clin. Microbiol*. 31: 3270–3274.

Akasaka Y, Ueda H, Takayama K, Machida Y and Nagai T. (1988). Preparation and evaluation of bovine serum albumin nanospheres coated with monoclonal antibodies. *Drug Des.Deliv*. 3: 85-97.

Arias-Bouda L M P, Nguyen L, Ho L, Kuijper S, Jansen H and Kolk A H J. (2000). Development of antigen detection assay for diagnosis of tuberculosis using sputum samples. *J. Clin. Microbl*. 38: 2278-2283.

Araj G F, Fahmawi B H, Chugh T D, Abu-Salim M. (1993). Improved detection of mycobacterial antigens in clinical specimens by combined enzymelinked immunosorbent assay. *Diagn. Microbiol. Infect. Dis*. 17: 119–127.

Ascierto P A, Simeone E, Sznol M, Fu Y X, Melero I.(2010). Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. *Semin. Oncol*. 37:508-516.

Azuma A, Yagita H, Matsuda H, Okumura K and Niitani H. (1992). Induction of intercellular adhesion molecule 1 on small cell lung carcinoma cell lines by gamma-interferon

enhances spontaneous and bispecific anti-CD3 x antitumor antibody-directed lymphokine activated killer cell cytotoxicity. *Cancer Res*. 52, 4890-94.

Banchuin N, Wongwajana S, Pumprueg U and Jearanaisilavong J. (1990). Value of an ELISA for mycobacterial antigen detection as a routine diagnostic test of pulmonary tuberculosis. *Asian Pac. J. Allergy Immunol*. 8: 5–11.

Bennion B J and Daggett V. (2003). The molecular basis for the chemical denaturation of proteins by urea. *Proc Natl Acad Sci U S A.* 100:5142-5147.

Bentley-Hibbert S I, Quan X, Newman T, Huygen K and Godfrey H P. (1999). Pathophysiology of antigen 85 in patients with active tuberculosis: antigen 85 circulates as complexes with fibronectin and immunoglobulin G. *Infect. Immunol.* 67:581–588.

Berry J D. (2005). Rational monoclonal antibody development to emerging pathogens, biothreat agents and agents of foreign animal disease: The antigen scale. *Vet J.* 170: 193-211.

Bhatnagar P K, Das D and Suresh M R. (2008). Sequential affinity purification of peroxidase tagged bispecific anti-sars cov antibodies on phenylboronic acid agarose. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* 863: 235-241.

Bioquant hCG ELISA kit (website last accessed on Feb 4th, 2011): http://www.bqkits.com/shopcart/images/product/BQ%20047F%20-%20HCG.pdf.

Bloom B R (1994) (Edited by). Tuberculosis: pathogenesis, protection, and control. (website last accessed on 4th Feb, 2011): http://books.google.ca/books?id=Nw44dW9d1D0C&pg=PA87&lpg=PA87&dq=tuberculosis+bacteria,+biosafety+level?&source=bl&ots=AcQeDOJkE1&sig=bz2332PMb9kK17

_pNWPvpbzUog8&hl=en&ei=fjhQTZOiFous8Ab9t6WBDw&sa=X&oi=book_result&ct=result&resnum=2&ved=0CB8Q6AEwAQ#v=onepage&q=tuberculosis%20bacteria%2C%20biosafety%20level%3F&f=false.

Boehme C, Molokova, Minja F, Geis S, Loscher T, Maboko L, Koulchin V and Hoelscher M. (2005). Detection of mycobacterial LAM with an antigen capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. *Trans. R. Soc. Trop. Med. Hyg.* 99: 893-900.

Bradford M M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annal. Biochem*. 72:248-54.

Brenner M.B, Trowbridge I S, and Strominger J L. (1985). Cross-linking of human T cell receptor proteins: association between the T cell idiotype beta subunit and the T3 glycoprotein heavy subunit. *Cell.* 40: 183-190.

Briken V, Porcelli SA, Besra GS and Kremer L. (2004). Mycobacterial lipoarabonamannan and related lipoglycans: from biogenesis to modulation of the immune response. *Mol. Microbiol.* 53: 391-403.

Burckhart T, Thiel M, Nishikawa H, Wüest T, Müller D, Zippelius A, Ritter G, Old L, Shiku H and Renner C. (2010). Tumor-specific crosslinking of GITR as costimulation for immunotherapy. *J. Immunother*. 33: 925-34.

Calabrò L, Danielli R, Sigalotti L and Maio M. (2010). Clinical studies with anti-CTLA-4 antibodies in non-melanoma indications. *Semin. Oncol.* 37: 460-467.

Canevari S, Stoter G, Arienti F, Bolis G, Colnaghi M I, Di Re E M, Eggermont A M, Goey S H, Gratama J W and Lamers C H, et al. (1995). Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. Natl. Cancer Inst*. 87: 1463-1469.

Cao Y, Christian S, Suresh M R. (1998). Development of a bispecific monoclonal antibody as a universal immunoprobe for detecting biotinylated macromolecules. *J. Immunol. Methods*. 220: 85-91.

Cao Y, Suresh M R. (1998). Bispecific antibodies as novel bioconjugates. *Bioconjugate chem*. 9: 635-644.

Cao Y, Suresh M R. (2000). Bispecific MAb aided liposomal drug delivery. *J. Drug Target.* 8: 257-266.

Chan E D, Reves R, Belisle J T, Brennan P J and Hahn W E. (2000). Diagnosis of tuberculosis by a visually detecTable immunoassay for lipoarabinomannan. *Am. J. Respir. Crit. Care Med.* 161:1713-1719.

Chatal J F, Faivre-Chauvet A, Bardies M, Peltier P, Gautherot E and Barbet J. (1995). Bifunctional antibodies for radioimmunotherapy. *Hybridoma* 14: 125-128.

Chiang C F, K Lo M, Rota P A, Spiropoulou C F and Rollin P E. (2010). Use of monoclonal antibodies against Hendra and Nipah viruses in an antigen capture ELISA. *Virol. J*. 7:115-122.

Chanteau S, Rasolofo V, Rasolonavalona T, Ramarokoto H, Horn C, Aurégan G and Marchal G (2000). 45/47 kilodalton (APA) antigen capture and antibody detection assays for the diagnosis of tuberculosis. *Int. J. Tuberc. Lung Dis*. 4: 377–383.

Chatterjee D, Khoo K H. (1998). Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology* 8: 113-120.

Chetanneau A, Barbet J, Peltier P, Le Doussal J M, Gruaz-Guyon A, Bernard A M, Resche I, Rouvier E, Bourguet P and Delaage M, et al. (1994). Pretargetted imaging of colorectal cancer recurrences using an 111Inlabeled bivalent hapten and a bispecific antibody conjugate. *Nucl. Med. Commun*. 15: 972-980.

Cho S N, Shin J S, Kim J D and Chong Y. (1990). Production of monoclonal antibodies to lipoarabinomannan-B and use in the detection of mycobacterial antigens in sputum. *Yonsei Med. J*. 31:333–338.

Cho SN. (2007). Current issues on molecular and immunological diagnosis of tuberculosis. *Yonsei Med. J.* 48: 347-359.

Choudhry V ans Saxena RK. (2002). Detection of *Mycobacterium tuberculosis* antigens in urinary proteins of tuberculosis patients. *Eur. J. Clin. Microbiol. Infect. Dis*. 21: 1–5.

Clarridge J E 3rd, Shawar R M, Shinnick T M and Plikaytis B B. (1993). Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J. Clin. Microbiol*. 31: 2049-2056.

Cockburn T A. (1964). The evolution and eradication of infectious disease. *Perspect Biol. Med*. 7:498-499.

Colebunders R and Bastian I. (2000). A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. *Int. J. Tuberc. Lung Dis*. 4:97–107.

Das D, Kammila S and Suresh MR. (2010). Development, characterization, and application of monoclonal antibodies against severe acute respiratory syndrome coronavirus nucleocapsid protein. *Clin Vaccine Immunol*. 17: 2033-2036.

Das D and Suresh M R. (2005). Producing Bispecific and Bifunctional Antibodies. *Methods Mol. Med*. 109: 329-346.

Davidow A, Kanaujia G V, Shi L, Kaviar J, Guo X, Sung N, Kaplan G, Menzies D and Gennar M L. (2005). Antibody profiles characteristic of *Mycobacterium tuberculosis* infection state. *Infect. Immun.* 73: 6846-6851.

De Gast G C, Van Houten A A, Haagen I A, Klein S, De Weger R A, Van Dijk A, Phillips J, Clark M and Bast B J. (1995). Clinical experience with CD3 x CD19 bispecific antibodies in patients with B cell malignancies. *J. Hematoth*. 4: 433-437.

De Lau W B, Van Loon A E, Heije K, Valerio D and Bast B J. (1989). Production of hybrid hybridomas based on HAT(s)-neomycin(r) double mutants. *J. Immunol. Methods*. 117: 1-8.

Dillehay L E, Mayer R, Zhang Y G, Shao Y, Song S Y, Mackensen D G, and Williams J R. (1995). Prediction of tumor response to experimental radioimmunotherapy with 90Y in nude mice. *Int. J. Radiat. Oncol. Biol. Phys*. 33: 417-427.

Dillon D C, Alderson M R, Day C H, Bement T, Campos-Neto A, Skeiky Y A W, Vedvick T, Badaro R, Reed S G and Houghton R. (2000). Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. *J. Clin. Microbiol*. 38: 3285-3290.

Doppalapudi V R, Huang J, Liu D, Jin P, Liu B, Li L, Desharnais J, Hagen C, Levin N J, Shields M J, Parish M, Murphy R E, Del Rosario J, Oates B D, Lai J Y, Matin M J, Ainekulu Z, Bhat A, Bradshaw C W, Woodnutt G, Lerner R A and Lappe R W. (2010). Chemical generation of bispecific antibodies. *Proc. Natl. Acad. Sci U S A*. 107: 22611-22616.

Ellner JJ. (1997). Regulation of the human immune response during tuberculosis. *J. Lab. Clin. Med.* 130: 469-475.

Escamilla L, Mancilla R, Glender W and Lopez-Marin L M. (1996). *Mycobacterium fortuitum* glycolipids for the serodiagnosis of pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med*. 154: 1864-1867.

Espitia C, Cervera I, González R, and Mancilla R. (1989). A 38-kD *Mycobacterium tuberculosis* antigen associated with infection. Its isolation and serologic evaluation. *Clin. Exp. Immunol*. 77: 373–377.

Ferguson J H and Schwarz M I. (2010). A 53-year-old man with dysphagia, anorexia, and night sweats. *Chest*. 138:1266-1270.

Fournier P, Aigner M and Schirrmacher V. (2010). Optimization studies for the coupling of bispecific antibodies to viral anchor molecules of a tumor vaccine. *Int. J. Oncol.* 37:1203-1217.

Fulcher DA and Basten A. (1997). B cell life span: A review. *Immunol and Cell Biol*. 75:446-455.

Gadikota R R, Callam C S Appelmelk B J, Lowary T L. (2003). Synthesis of oligosaccharide fragments of mannosylated lipoarabinomannan appropriately functionalized for neoglycoconjugate preparation. *J. Carbohydr. Chem.* 22: 459–480.

Gennaro ML. (2000). Immunological diagnosis of tuberculosis. *Clin. Infect. Dis*. 30(Suppl): S243-S246.

Gennaro ML. (2005). Serological tests for TB: is there hope? *Int. J. Tuber. Lung Dis.* 9: S18.

Glennie M J, McBride H M, Worth A T and Stevenson G T. (1987). Preparation and performance of bispecific F(ab¢ gamma) 2 antibody containing thioether-linked Fab¢ gamma fragments. *J. Immunol*. 139: 2367-2375.

Golden M P and Vikram H R. (2005). Extrapulmonary tuberculosis: an overview. *Am. Fam. Physician.* 72: 1761-1768.

Gómez B L, Figureueroa J I, Hamilton A J, Ortiz B, Robledo M A, Hay R J, and Restrepo A. (1997). Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. *J. Clin. Microbiol*. 35: 3278–3283.

Granich R, Akolo C, Gunneberg C, Getahun H, Williams P and Williams B. (2010). Prevention of tuberculosis in people living with HIV. *Clin. Infect. Dis*. 50 Suppl 3:S215-S222.

Gutierrez M C, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, Supply P and Vincent V. (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS. Pathol*. 1:e5.

Guttikonda S, Tang X L, Yang B M, Armstrong G D and Suresh MR. (2007). Monospecific and bispecific antibodies against E. coli O157 for diagnostics. *J . Immunol. Methods*. 327: 1–9.

Hamasur B, Bruchfeld J, Haile M, Pawlowski A, Bjorvatn B, Källenius G and Svenson S B. (2001). Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. *J. Microbiol. Methods*. 45:41–52.

Hendrickson R C, Douglass J F, Reynolds L D, McNeill P D, Carter D, Reed S G and Houghton R L. (2000). Mass spectrometric identification of Mtb81, a novel serological marker for tuberculosis. *J. Clin. Microbiol*. 38: 2354-2361.

Holliger P, Prospero T, and Winter G. (1993). "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U.S.A*. 90: 6444-6448.

Hunter S W, Gaylord H and Brrennan P J. (1986). Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tuberle bacilli. *J. Biol. Chem.* 261: 12345-12351.

Inderlied CB, Kemper CA, Bermúdez LE. 1993. The *Mycobacterium avium* complex. *Clin. Microbiol. Rev*. 6: 266-310.

Irmen K E and Kelleher J J. (2000). Use of monoclonal antibodies for rapid diagnosis of respiratory viruses in a community hospital. *Clinl. Diag. Lab. Immunol.* 7: 396-403.

Jozefowski S, Sobota A and Kwiatkowska K. (2008). How *Mycobacterium tuberculosis* subverts host immune responses. *Bio Essays*. 30: 943-954.

Jung G, Freimann U, Von Marschall Z, Reisfeld R A, and Wilmanns W. (1991). Target cell-induced T cell activation with bi- and trispecific antibody fragments. *Eur. J. Immunol*. 21:2431-2435.

Kaur D, Lowary T L, Visa D V, Crick D C and Brennan P J. (2002). Charecterization of the epitope of anti-lipoarabinomannan antibodies as the terminal hexaarabinofuranosyl motif of mycobacterial arabinans. *Microbiology* 148: 3049-3057

Kammila S, Das D, Bhatnagar P K, Sunwoo H H, Zayas-Zamora G, King M and Suresh M R. (2008). A rapid point of care immunoswab assay for SARS-CoV detection. *J. Virol Methods.* 152: 77-82.

Kindt T J, Goldsby R A and Osborne B A. (2007). Kuby immunology. 6th Edition.

Knuth A, Bernhard H, Jager E, Wolfel T, Karbach J, Jaggle C, Strittmatter W and Meyer zum Buschenfelde K H. (1994). Induction of tumour cell lysis by a bispecific antibody recognising epidermal growth factor receptor (EGFR) and CD3. *Eur. J. Cancer* 30A: 1103-1107.

Kohler G and Milstein C. (1975). Continous cultures of fused secreting antibody of predefined specificity. *Nature.* 256: 495-497.

Kranenborg M H, Boerman O C, Oosterwijk-Wakka J C, de Weijert M C, Corstens F H, and Oosterwijk E. (1995). Development and characterization of anti-renal cell carcinoma x antichelate bispecific monoclonal antibodies for two-phase targeting of renal cell carcinoma. *Cancer Res*. 55, 5864-55867.

Kranz D M, Gruber M, and Wilson E R. (1995). Properties of bispecific single chain antibodies expressed in *Escherichia coli*. *J. Hematother*. 4: 403-408.

Kreutz F T and Suresh M R. (1997). Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. *Clin. Chem* 43:649–656.

Kreutz, F.T, Xu, D, Suresh M.R. (1998). A new method to generate quadromas by electrofusion and FACS sorting. *Hybridoma.* 17: 267-273.

Laal S and Skeiky Y A W. (2004). Immune based methods of diagnosis. In: Cole ST, Gicquel B, McMurray DN, Eisenach KD, Jacobs WR, editors. tuberculosis and the tubercle bacillus. Second Edition. *Washington DC: ASM Press*. pp. 71-84.

Laemmli U K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227: 680-685.

Landowski C P, Godfrey H P, Bentley-Hibbert S I, Liu X, Huang Z, Sepulveda R, Huygen K, Gennaro M L, Moy F H, Lesley S A, Haak-Frendscho M. (2001). Combinatorial use of antibodies to secreted mycobacterial proteins in a host immune system-independent test for tuberculosis. *J. Clin. Microb*. 39: 2418–2424.

Leemans J C, Juffermans N P, Florquin S, Van Rooijen N, Vervoordeldonk M J, Verbon A, Van Deventer S J H and Van der Poll T. (2001). Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. *J. Immunol*. 166: 4604–4611.

Lipman N S, Jackson L R, Trudel L J and W-Garcia F. (2005). Monoclonal versus polyclonal antibodies : distinguishing characteristics, applications and information resources. *Inst Lab. Anim. Resea. J.* 46: 258-268.

Liu F, Guttikonda S and Suresh M R. (2003). Bispecific monoclonal antibodies against a viral and an enzyme: utilities in ultrasensitive virus ELISA and phage display technology. *J. Immunolo. Methods* 274 : 115– 127.

Lodes M J, Dillon D C, Mohamath R, Day C H, Benson D R, Reynolds L D, McNeill P, Sampaio D P, Skeiky Y A W, Badaro R, Persing D H, Reed S J and Houghton R L. (2001). Serological expression cloning and immunological evaluation of Mtb48, a novel *Mycobacterium tuberculosis* antigen. *J. Clin. Microbiol*.39: 2485-2493.

Mabey D, Peeling R W, Ustianowski A and Perkins M D. (2004). Diagnostics for the developing world. *Nat. Rev. Microbiol.* 2:231-240.

Mabry R and Snavely M. (2010). Therapeutic bispecific antibodies: The selection of stable single-chain fragments to overcome engineering obstacles. *IDrugs*. 13:543-549.

Madariaga M G, Jalali Z, and Swindells S (2007). Clinical utility of interferon gamma assay in the diagnosis of tuberculosis. *J. Am. Board. Fam. Med.* 20:540 –547.

Menon P K, Kapila K and Ohri V C. (2000). Recent advances in tuberculosis diagnostic techniques. *MJAFI.* 56: 143-148.

Milestein C and Cuello A C (1983). Hybrid hybridomas and their use in immunohistochemistry. *Nature*. 305: 537-540.

Miller R A, Dissanayake S and Buchanan T M (1983). development of an enzyme-linked immunosorbent assay using arabinomannan from *Mycobacterium smegmatis*: a potentially useful screening test for the diagnosis of incubating leprosy. *Am. J. Trop. Med. Hyg.* 32: 555-564.

Mitchison D A (1979). Basic Mechanisms of Chemotherapy. *Chest.* 76:771-781.

Niikura M, Ikegami T, Saijo M, Kurane I, Miranda M E and Morikawa S. (2001). Detection of ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. *J. Clin. Microbiol*. 39: 3267-3271.

Nisonoff A and Rivers M M. (1961). Recombination of a mixture of univalent antibody fragments of different specificity. *Arch. Biochem. Biophys*. 93: 460-462.

Ohta S, Tsukamoto H, Watanabe K, Makino K, Kuge S, Hanai N, Habu S and Nishimura T. (1995). Tumorassociated glycoantigen, sialyl Lewis(a) as a target for bispecific antibody-directed adoptive tumor immunotherapy. *Immunol. Lett.* 44, 35-40.

Online text book of bacteriology: website last accessed on 19 October 2010: http://www.textbookofbacteriology.net/tuberculosis.html.

Pai M, Kalantri S and Dheda K. (2006). New tools and emerging technologies for the diagnosis of tuberculosis: part II. Active tuberculosis and drug resistance. *Expert Rev. Mol Diagn.* 6:423-432.

Palma-Nicolás JP and Bocanegra-García V. (2007). Innovative Strategies to diagnose and monitor tuberculosis patients. *Arch. Bronconeumol*. 43: 225-232.

Patel VB, Singh R, Connolly C, Kasprowicz V, Zumla A, Ndungu T and Dheda K. (2010). Comparison of a clinical prediction rule and a LAM antigen-detection assay for the rapid diagnosis of TBM in a high HIV prevalence setting. *PLoS One*. 5:e15664.

Perkins M D and Jane Cunningham J. (2007). Facing the Crisis: Improving the Diagnosis of tuberculosis in the HIV era. *J. Infect. Dis*. 196: S15–S27.

Pitchenik A E, Cole C, Russell B W, Fischl M A, Spira T J and Snider DE Jr. (1984). Tuberculosis, atypical mycobacteriosis and acquired immunodefienciency syndrome among Haitian and non-Haitian patients in South Florida. *Ann. Int. Med*. 101: 641-645.

Prinzis S, Chatterjee D and Brennan P J. (1993). Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. *J. Gen. Microbiol*. 139: 2649-2658.

Rademacher C, Shoemaker G K, Kim H, Zheng R B, Taha H, Liu C, Nacario R C, Schriemer D C, Klassen J S, Peters T and Lowary T L. (2007). Ligand specificity of CS-35, a monoclonal antibody that recognizes mycobacterial lipoarabinomannan: a model system for oligofuranoside-protein recognition. *J Ame. Chem. Soc*. 129: 10489-10502.

Rothschild B M, Martin L D, Lev G, Bercovier H, Bar-Gal G K, Greenblatt C, Donoghue H, Spigelman M and Brittain D. (2001). *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present. *Clin. Infect. Dis*. 33:305–311

Sada E, Ferguson L E and Daniel T M. (1990). An ELISA for the serodiagnosis of tuberculosis using a 30,000-Da native antigen of *Mycobacterium tuberculosis*. *J. Infect. Dis.* 162: 928-931.

Sada E , Aguilar D, Torres M and Herrera T. (1992). Detection of lipoarabinomannan as a diagnostic test for tuberculosis. *J Clin. Microbiol*. 30: 2415-2418.

Schmidt, M., Hynes, N. E., Groner, B., and Wels, W. (1996). A bivalent single-chain antibody-toxin specific for ErbB-2 and the EGF receptor. *Int. J. Cancer.* 65: 538-546.

Schuhmacher J, Klivenyi G, Matys R, Stadler M, Regiert T, Hauser H, Doll J, Maier-Borst W, and Zoller M. (1995). Multistep tumor targeting in nude mice using bispecific antibodies and a gallium chelate suitable for immunoscintigraphy with positron emission tomography. *Cancer Res*. 55: 115-123.

Segal D M, Qian J H, Garrido M A, Perez P, Winkler D F, Wunderlich J R, Snider D P, Valdayo M J and Titus J A. (1988). Targeting of cytotoxic cells against tumors with heterocross-linked, bispecific antibodies. *Princess Takamatsu Symp*. 19:323-331.

Shahhosseini S, Das D, Qiu X, Feldmann H, Jones SM and Suresh MR. (2007). Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. *J. Virol. Methods*. 143: 29–37.

Shahhosseini S, Guttikonda S, Bhatnagar P and Suresh MR. (2006). Production and characterization of monoclonal antibodies against shope fibroma virus superoxide dismutase and glutathione-s-transferase. *J. Pharm. Pharmaceut. Sci*. 9:165-168.

Shalaby M R, Shepard H M, Presta L, Rodrigues M L, Beverley P C, Feldmann M, and Carter P. (1992). Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J. Exp. Med.* 175: 217-225.

Sharkey R M, Rossi E A, McBride W J, Chang C H and Goldenberg D M. (2010). Recombinant bispecific monoclonal antibodies prepared by the dock-and-lock strategy for pretargeted radioimmunotherapy. *Semin. Nucl. Med.* 40: 190-203.

Sharma S K and Mohan A. (2004). Extrapulmonary tuberculosis. *Indian J. Med. Res.* 120: 316-353.

Sharma S K, Mohan A and Kadhiravan T. (2005). HIV-TB co-infection: Epidemiology, diagnosis & management. *Indian J. Med. Res* 121: 550-567.

Silva V M C, Kanaujia G, Gennaro M L and Menzies D. (2003). Factors associated with humoral response to ESAT-6, 38 kDa and 14 kDa in patients with a spectrum of tuberculosis. *Int. J. Tuberc. and Lung Dis*. 7: 478-484.

Spencer JS, Kim HJ, Wheat WH, Chatterjee D, Balagon MV, Cellona RV, Tan EV, Gelber R, Saunderson P, Duthie MS, Reece ST, Burman W, Belknap R, Mac Kenzie WR, Geluk A, Oskam L, Dockrell HM, Brennan PJ; IDEAL Consortium. (2011). Analysis of antibody responses to Mycobacterium leprae phenolic glycolipid I, lipoarabinomannan, and recombinant proteins to define disease subtype-specific antigenic profiles in leprosy. Clin Vaccine Immunol. 18:260-267.

Steingart K R, Ng V, Henry M, Hopewell P C, Ramsay A, Cunningham J, Urbanczik R, Perkins M D, Aziz M A and Pai M. (2006). Sputum processing methods to improve the

sensitivity of smear microscopy for tuberculosis: a systematic review. *Lancet Infect. Dis.* 6: 664-674.

Stop TB Partnership (website last accessed on 15th July, 2010) http://www.stoptb.org/assets/documents/global/plan/SP%20Stop%20TB%20Dia%20WG%20-FINAL-Dec2005.pdf.

Strohmeier G R and Fenton M J. (1999). Roles of lipoarabonomannan in the pathogenesis of tuberculosis. *Microb. Infect*. 1:709-717.

Sumi M G, Mathai A, Reuben S, Sarada C and Radhakrishnan V V. (2002). Immunocytochemical method for early laboratory diagnosis of tuberculous meningitis. *Clin. Diagn. Lab. Immunol.* 9:344–347.

Suresh M R, Cuello A C, and Milstein C. (1986a). Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays. *Proc. Natl. Acad. Sci. U.S.A*. 83, 7989-7993.

Suresh M R, Cuello A C, and Milstein C. (1986b). Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol*. 121: 210-228.

Syrigos K N, Deonarian D P and Epenetos A A. (1999). Use of monoclonal antibodies for the diagnosis and treatment of bladder cancer. *Hybridoma.* 18: 219-224.

Tang X L, Peppler M S, Irvin R T and Suresh M R. (2004). Use of bispecific antibodies in molecular velcro assays whose specificity approaches the theoretical limit of immunodetection for *Bordetella pertusis*. *Clin. Diag. Lab. Immunol*. 11: 752-757.

The Tuberculosis Trials Consortium (2002). Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. *Lancet*. 360:528-34.

TDR publication 2006: (website last accessed on 4th Feb, 2011) http://www.who.int/tdrold/publications/publications/pdf/tbdi/tbdi_chap2.pdf.

TDR publication 2006 A: (last accessed on 4th Feb, 2011) http://www.who.int/tdrold/publications/publications/pdf/tbdi/tbdi_chap3.pdf.

Tessema T A, Hamasur B, Bjun G, Svenson S and Bjorvatn B. (2001). Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre. *Scand. J. Infect. Dis*. 33:279–284.

Tessema T A, Bjune G, Hamasur B, Svenson S, Syre H and Bjorvatn B. (2002). Circulating antibodies to lipoarabinomannan in relation to sputum microscopy, clinical features and urinary anti-lipoarabinomannan detection in pulmonary tuberculosis. *Scand. J. Infect. Dis*. 34:97–103.

Tessema T A, Bjune G, Assefa G, Svenson S, Hamasur B and Bjorvatn B. (2002b). Clinical and radiological features in relation to urinary excretion of lipoarabinomannan in Ethiopian tuberculosis patients. *Scand. J. Infect. Dis.* 34:167–171.

The Tuberculosis Trials Consortium (2002). Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. *Lancet.* 360: 528–534.

Thirion S, Motmans K, Heyligen H, Janssens J, Raus J, and Vandevyver C. (1996) Mono- and bispecific singlechain antibody fragments for cancer therapy. *Eur. J. Cancer Prev.* 5: 507-511.

Todar's online textbook of bacteriology: (website last accessed on 4th Feb, 2011) http://www.textbookofbacteriology.net/resantimicrobial.html

Van Dijk J, Tsuruo T, Segal D M, Bolhuis R L, Colognola R, van de Griend R J, Fleuren G J, and Warnaar S O. (1989). Bispecific antibodies reactive with the multidrug-resistance-related glycoprotein and CD3 induce lysis of multidrug-resistant tumor cells. *Int. J. Cancer*. 44: 738-743.

Van Dijk J, Zegveld S T, Fleuren G J and Warnaar S O. (1991). Localization of monoclonal antibody G250 and bispecific monoclonal antibody CD3/G250 in human renalcell carcinoma xenografts: relative effects of size and affinity. *Int. J. Cancer* 48: 738-743.

Vasudevachari M B, Salzman N P, Woll D R, Mast C, Uffelman K W, Toedter G, Hoefheinz D, Metcalf J A and Lane H C. (1993). Clinical utility of an enhanced human immunodeficiency virus type 1 p24 antigen capture assay. *J. Clin. Immunol*. 13: 185-192.

Waldmann T A. (2003). Immunotherapy: past, present and future. *Nat. Med*. 9: 269-277.

Wallis R S, Perkins M, Phillips M, Joloba M, Demchuk B, Namale A, Johnson J L, Williams D, Wolski K, Teixeira L, Dietze R, Mugerwa R D, Eisenach K and Ellner J J (1998). Induction of the antigen 85 complex of M. tuberculosis in sputum: a determinant of outcome in pulmonary tuberculosis treatment. *J Infect Dis.* 178:1115-1121.

Wang W W, Das D, Tang X L, Budzynski W and Suresh M R. (2005). Antigen targeting to dendritic cells with bispecific antibodies. *J Immunol. Methods.* 306: 80-92.

Wang W W, Das D and Suresh M R. (2009). A versatile bifunctional dendritic cell targeting vaccine vector. *Mol. Pharmaceu*. 6: 158–172.

Wecker L, Crespo L M, Dunaway G, Faingold C and Watts S. (2010). Brody's human pharmacology molecular to clinical. (5^{th} edition).

WHO infectious disease online report on, (website last accessed on 22nd June 2010). http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_AnnexA.pdf.

WHO global TB report 2009: (website last accessed on 15th July 2010.) http://www.who.int/tb/publications/global_report/2009/pdf/report_without_annexes.pdf .

WHO global TB report 2010: (website last accessed on 2nd Feb 2011.) http://whqlibdoc.who.int/publications/2010/9789241564069_eng.pdf

WHO tuberculosis factsheet (website last accessed on February 3, 2011): http://www.who.int/mediacentre/factsheets/fs104/en/.

Wikipedia website: (last accessed on March 15, 2011): http://en.wikipedia.org/wiki/Monoclonal_antibody_therapy

Winter G. and Milstein C. (1991). Man-made antibodies. *Nature* 349: 293-299.

Yager P, Domingo G J and Gerdes J. (2008). Point-of-care diagnostics for global health. *Annu. Rev. Biomed. Eng.* 10: 107-44.